Characterization of beta-nicotyrine-mediated inactivation of cytochrome P450 2A6

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Sharon E. Murphy, Advisor

April 2013

Acknowledgements

My first and most sincere thanks go to my thesis advisor, Dr. Sharon E. Murphy. You have offered me sound advice and careful guidance. Thank you for your time, energy and patience.

I would like to express my deep gratitude and respect to Dr. Lisa Peterson, Dr. Linda von Weymarn, Dr. Peter Villalta, Brock Matter, Dr. Aaron Titelbaum and Dr. Rory Remmel. Each of you has offered valuable input that contributed to my thesis project.

I would like to thank my fellow graduate students Martin Phillips, Ania Urban, Nicole Skinner, Lauren Beech, IlyaTikh, Josh Baller, and Adam Zarth; struggling and learning alongside each of you has been an amazing journey.

To my Murphy Lab co-workers, thank you for sharing your expertise and for making the lab a friendly environment.

To everyone on the 7th floor of the Cancer Center, thank you for your friendship and willingness to share your extensive knowledge on every possible subject.

Finally, to my family, thank you for your unending support and the joy you bring to my life. You constantly inspire me to work hard, persist through difficult moments and embrace the world a sense of humor.

Dedication

For my family, who offer me unconditional love and support.

Abstract

Nicotine, the primary addictive compound in cigarettes, is metabolized in humans by cytochrome P450 2A enzymes. The hepatic enzyme responsible for the metabolism of nicotine in smokers is P450 2A6. P450 2A13, which shares 94% primary sequence homology with P450 2A6, also catalyzes the metabolism of nicotine and is present in the lung. Loss of P450 2A activity is correlated with modified smoking behavior and addiction. Inhibition of these enzymes decreases nicotine metabolism and may be of benefit in smoking cessation.

This thesis investigates tobacco-related molecules that may impact P450 2A function and is presented in three parts. In the first, the potential inhibitory potency of (-)-menthol, (R)-(+)-menthofuran, and β-nicotyrine of both P450s 2A6 and 2A13 are investigated. All three compounds inhibit P450 2A6 and 2A13 activity. In addition, menthofuran and β-nicotyrine mediate mechanism-based inactivation of P450 2A6 but not 2A13. Second, the P450 2A6 and P450 2A13-mediated metabolism of β-nicotyrine is studied and three metabolites are identified. β-nicotyrine is readily turned over by both P450 2A6 and P450 2A13 as indicated by the calculated K_m (4.4μM and 5.0μM, respectively) and V_{max} (21 and 37 pmol product/min/pmol, respectively) values. Also in the second section, β-nicotyrine is shown to be a metabolite of P450 2A6-mediated nicotine metabolism. In the last section, attempts to identify a covalent adduct that would result from menthofuran or β-nicotyrine-mediated inactivation are presented, but these are largely unsuccessful.

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Chapter 1

Introduction

There has been extensive research on tobacco and its effects on human health. It is well established that tobacco causes cancer and other diseases and major players in disease processes have been identified. It is also known that tobacco use results in addiction to nicotine and this addiction propels use which leads to cancer (Figure 1-1). This thesis is focused on the study of molecular interaction between less well studied tobacco-related molecules and the two enzymes that are implicated in nicotine clearance and procarcinogen activation. Although much of the research presented is mechanistic in nature, the big-picture remains focused on how to reduce human disease caused by tobacco use, which includes encouraging the discontinuation of tobacco use. Thus, a summary covering general tobacco knowledge is provided below.

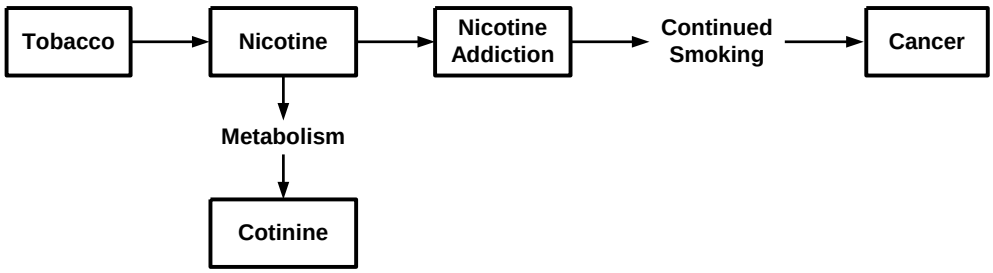


Figure 1-1. Role of Nicotine Metabolism in Tobacco-Related Cancers.

1.1 Tobacco use and health consequences

Tobacco use is prevalent across the globe and a common method of tobacco use is cigarette smoking. It has been estimated that worldwide there are 1 billion males who smoke and 250 million females[1]. The World Health Organization has indicated that the tobacco pandemic is moving from Western countries to developing nations and has estimated that about 80% of the people who die from smoking now live in low and middle income countries[1;2].

Despite this shift, tobacco use persists in the United States. Approximately 46.6 million U.S. adults smoke cigarettes[3]. Although there was a steady decrease in the percentage of adult smokers in the US from 1965 (42%) until 2004 (21%), the value has plateaued near 20% where it remains to the present day (Figure 1-2). In 2009, an estimated 20% of the teenage population in the United States were self-reported smokers, indicating that it is likely there will be no further decline in smoking in the next generation [4]. In addition to those who smoke, many Americans, including 54% of children aged 3–11 years, are exposed to secondhand smoke [5].

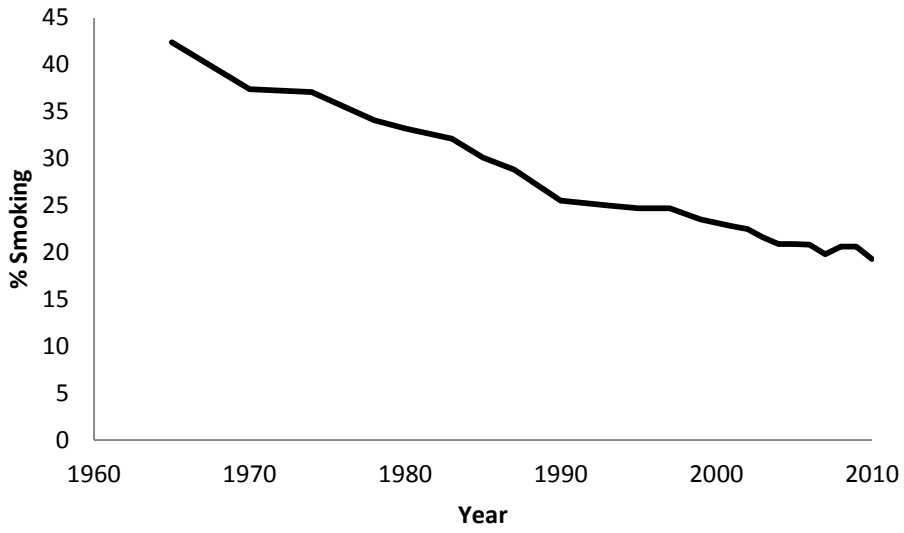


Figure 1-2. Percentage of American adults who are current cigarette smokers [6].

There are many negative health outcomes that result from tobacco use and exposure to secondhand tobacco smoke. Lung cancer is the most well-known but there is sufficient evidence that cancers of the lung, larynx, nasal cavity, oral cavity, esophagus, liver, pancreas, bladder, cervix and leukemia are associated with tobacco use[7]. Additionally, coronary heart disease, stroke, chronic obstructive lung diseases (such as chronic bronchitis and emphysema) and reproductive problems are linked to tobacco use[7]. In U.S. fifty percent of smokers die as a direct consequence of their tobacco addiction, this sums to an estimated 400,000, or one in every five deaths, every year [8;9]. Health risks from smoking are not limited to the smoker; it has been estimated that worldwide 600,000 people die each year from exposure to secondhand tobacco smoke[1;10]. Tobacco is considered a major preventable cause of morbidity and mortality, but it is a

challenging problem. Preventing or successfully treating addiction to tobacco products is imperative.

1.2 Tobacco regulation and smoking cessation

The United States has a long history of using state and federal legislation in attempts to reduce tobacco use and secondhand smoke exposure in the population. Efforts are typically targeted toward the regulation of tobacco sales, marketing, and use. Federal legislation include acts that require labeling cigarette packages as hazardous (1965), obligate reports of any additives used in manufactured tobacco products (1984) and the prohibition of smoking on airplanes (1989) and in clinics that treat women and children (1993)[11]. State legislation has mandated a smoking ban in 22 states, however the specifics vary from state to state[12]. Current approaches to decrease tobacco use include smoke-free legislation, taxes on tobacco products to raise prices, bans on product promotion, education outreach, warning labels and accessible quit programs. In 2009, the Family Smoking Prevention and Tobacco Control Act granted the Food and Drug Administration (FDA) the authority to regulate tobacco products. Under this act, a ban on cigarettes containing certain characterizing flavors went in to effect. This ban did not include menthol, a common cigarette flavoring that provides a sort of coolness in the mouth while smoking. Menthol cigarettes are preferred by 75% of African American smokers but only 23% of Caucasian smokers [13]. African Americans have higher lung cancer incidence rates (74.7 per 100,000) than any other ethnic or racial group, including Caucasians (64.4 per 100,000) [13;14]. The correlation generated controversy since menthol

is reported to modify nicotine metabolism and has thus been the focus of recent FDA regulation [15-17].

Despite governmental efforts, there are several challenges to decreasing tobacco use in the U.S. The tobacco industry has strong roots in the global economy; Philip Morris, the largest US-based cigarette company, has yearly revenues of around 44 billion dollars and a high trade surplus, resulting in it being a high US tax-payer [18]. As a result, tobacco companies have had influence in the negotiations regarding tobacco legislation and are actively developing new products and strategies to promote tobacco use that circumvent the resulting policies.

Anti-smoking policies and increased awareness of health risks have resulted in decreased tobacco use in the United States though some demographics maintain higher rates of smoking. The percentage of smoking American adults from the population that is at or above the poverty level (18%) is substantially lower than from the population that is below the poverty level (29%) [3]. Additionally, those with higher levels of education tend to smoke less (Figure 1-3) [3]. Those of a lower economic status are less likely to have access to healthcare and are thus likely to be affected more by tobacco-related health problems.

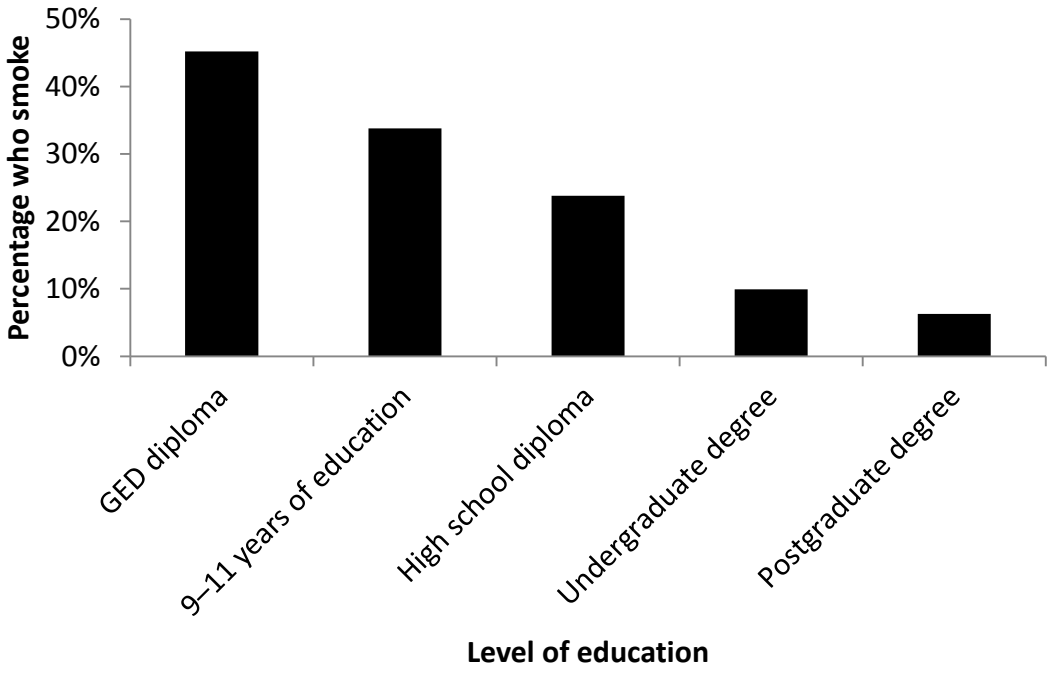


Figure 1-3. Percentage of adult smoking by education level[3].

From 2000 to 2004, cigarette smoking was estimated to be responsible for $193 billion in annual health-related economic losses in the United States [19]. Tobacco also creates economic costs that extend beyond the direct cost of related illness and productivity losses, including health care expenditures from active and passive smokers, employee absenteeism, reduced labor productivity, fire damage due to careless smokers, increased cleaning costs, and widespread environmental damage [19]. The health and economic consequences of tobacco use are entirely preventable.

Quitting tobacco simultaneously reduces health risks and produces long-term health benefits [20]. Despite the potential mitigating effects of quitting, many continue to use tobacco. The Center for Disease Control (CDC) informs us that in 2010, 69% of

adult smokers reported that they would like to quit, 52% actually made a quit attempt but only 6.2% were successful in their attempt [21]. These quitting statistics are explained by research that compares the strength of addiction to nicotine, the addictive compound in tobacco, to heroin, cocaine, or alcohol [22].

1.3 Relevant components of tobacco

There are over 4,000 chemicals in tobacco smoke. This mix contains many known or suspected human carcinogens and toxic agents. The FDA has recognized 93 of these compounds as harmful and 69 are known carcinogens [23]. The way to minimize exposure to these harmful compounds is to discontinue tobacco use. However many users struggle to quit because of the strength of nicotine addiction. Therefore, the study of compounds that cause or mitigate damaging effects of tobacco or influence addiction are of particular interest.

1.3.1 Nicotine

Nicotine is the addictive agent in tobacco products and it is the primary reason why people continue their use [24]. Found in the leaves of the *Solanaceae* family of plants, which include tomatoes, potatoes and tobacco, nicotine functions as a natural pesticide that is toxic to insects. Most of these plants have very low levels of nicotine (4-100 ng/g)[25]. In tobacco plant leaves, however, nicotine is up to 3% (30mg/g) of the dry weight.

The effects of nicotine are related to blood levels of nicotine, the kinetics of exposure and the resulting psychoactive effect. Nicotine is an alkaloid that acts as an acetylcholine analogue in humans. One cigarette contains 10-14 mg of nicotine and a smoker inhales an estimated 1mg per cigarette smoked [26]. When tobacco smoke reaches the lungs, nicotine is rapidly absorbed and reaches the brain in 10-20 seconds. In the brain, nicotine binds to nicotinic cholinergic receptors, causing complex effects that are mediated by dopinergic and nondopinergic neurons [27;28]. Blood concentrations of nicotine rise quickly during smoking and peak at the completion of the cigarette. Peak nicotine concentrations provide the smoker with sought effects, typically relaxation, reduced stress and pleasant mood [28]. The rapid rise in nicotine levels permits the smoker to titrate the level of nicotine during smoking [29;30]. Though smokers seek out the favorable effects that occur at peak nicotine levels, smoking behavior more closely corresponds to maintaining a threshold concentration in the blood [30]. Maintaining a nicotine threshold mitigates withdrawal symptoms, which include nervousness, restlessness, irritability and anxiety. Supporting this idea of nicotine threshold maintenance, cigarette smokers switching from a higher to a lower-yield cigarette or reducing the number of cigarettes smoked, will compensate, i.e., will change the smoking pattern to gain more nicotine [31-33].

The amount of circulating nicotine is a function of the dose consumed and the rate of elimination [26]. The primary route of nicotine elimination is through hepatic metabolism, though some metabolism occurs in other tissues including the lungs and kidney [29]. Nicotine's conversion to cotinine through 5'-oxidation is the major metabolic

pathway. None of the major metabolites have psychoactive properties. In most people nicotine-C-oxidation to generate cotinine is the major metabolic pathway. In most smokers, about 70–80% of the nicotine dose is excreted as cotinine and its metabolites (Figure 1-4) [34]. Ninety percent of a systemic dose of nicotine can be accounted for as nicotine and metabolites in the urine [35]. Thirty to 40% is excreted as *trans*-3'-hydroxycotinine, a subsequent oxidation product of cotinine. Unchanged nicotine accounts for only 8-10% of the dose [34]. The average half-life of nicotine is two hours, however the rate of metabolism and distribution of metabolites vary among individuals [36].

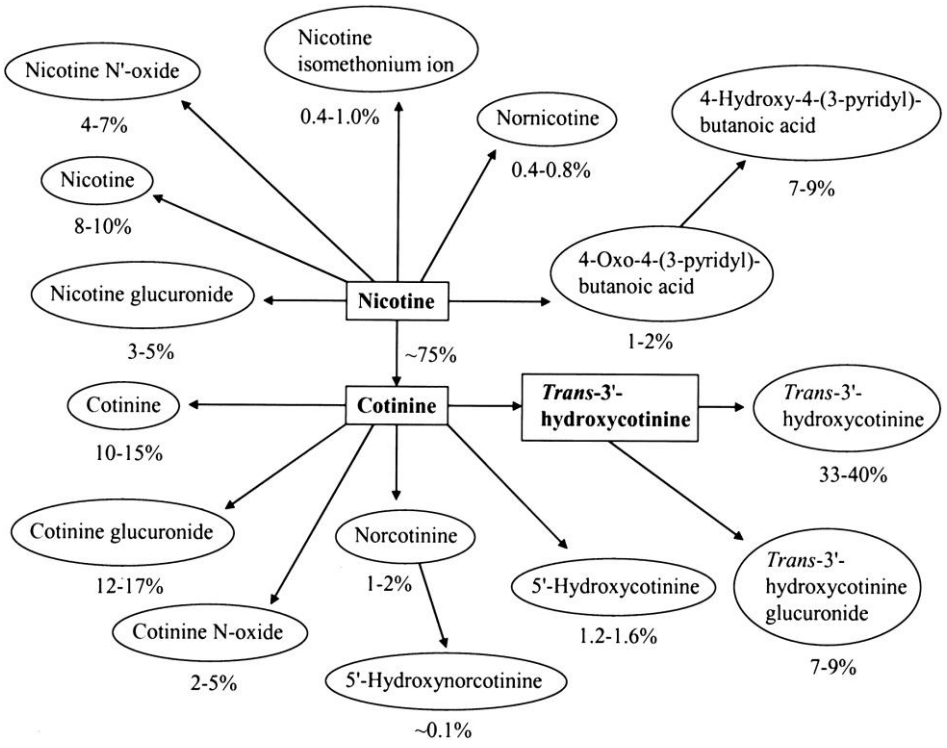


Figure 1-4. Quantitative scheme of nicotine metabolism. Based on estimates of average excretion of metabolites as percent of total urinary nicotine[26].

1.3.2 4-(methylnitrosamino)- 1-(3-pyridyl)-1-butanone (NNK)

Among the 69 known tobacco carcinogens is the tobacco specific human lung-carcinogen, NNK [37]. NNK is structurally related to nicotine, and is formed during the tobacco curing process (Figure 1-5). Considerable evidence favors NNK as major etiological factors in lung cancer, particularly important in the induction of adenocarcinoma [38].

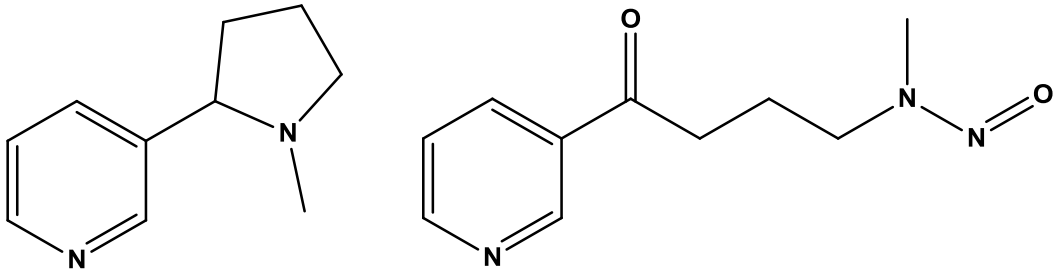


Figure 1-5: Structure of nicotine and NNK

Levels of NNK are abundant; albeit 10,000-fold lower than nicotine (433-733 ng NNK/cigarette) [37;38]. Metabolism of NNK is necessary for carcinogenesis, requiring hydroxylation alpha to the *N*-nitroso moity [39]. This hydroxylation is efficiently catalyzed by an enzyme present in the lung suggesting that tissue specific activation of NNK contributes to the heightened risk of lung cancer among smokers.

1.3.3 1-methyl-2-(3-pyridyl) pyrrole (β-nicotyrine)

Although nicotine is the predominant tobacco alkaloid, there are a handful of other 'minor' alkaloids present in tobacco. Included in this list are anatabine, anabasine, mysomine, and β-nicotyrine. The structures of the aforementioned alkaloids are similar to nicotine. β-nicotyrine differs from nicotine only in that the pyrrolidine ring has been aromatized by the loss of four hydrogen atoms (Figure 1-6).

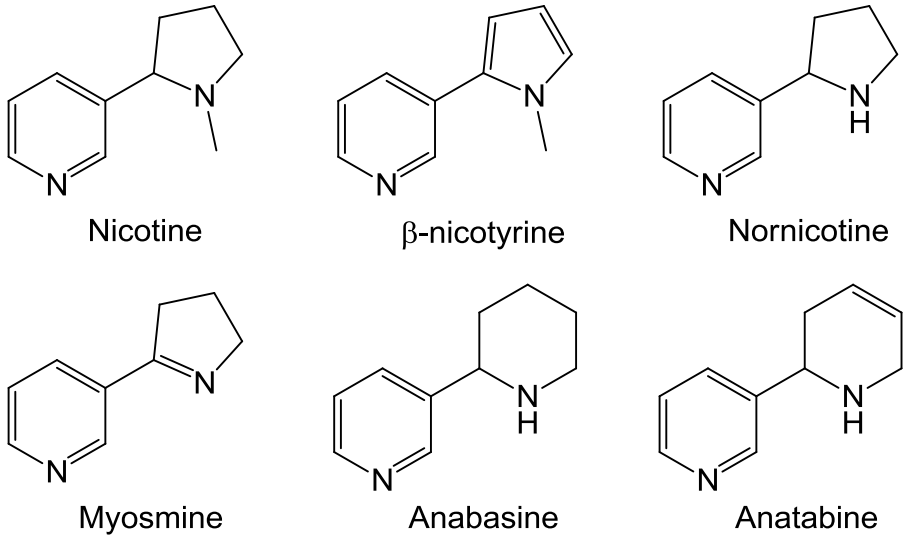


Figure 1-6: Nicotine and minor tobacco alkaloids.

β-Nicotyrine is present in tobacco and is a product of thermal decomposition of nicotine [40;41]. It is speculated that β-nicotyrine is generated during the curing process of tobacco as well as the burning of a cigarette. The amount of β-nicotyrine in tobacco smoke has been reported to range from 4-40 µg per cigarette compared to 2-20 µg for

anatabine and 0.8 to 2.3 mg for nicotine [42;43]. β-nicotyrine has also been detected in environmental tobacco smoke and in the E-cigarette [44;45].

β-nicotyrine is present in tobacco and tobacco smoke but the physiological implications, if any remain unknown. In animal tissues, β-nicotyrine is reported to possess some weak nicotine-like pharmacological activities [46]. No work has been done looking at the physiological effects of β-nicotyrine in humans.

Little is known of β-nicotyrine metabolism in smokers. There is one published study in which β-nicotyrine (a 2 mg oral dose) was given to humans [47]. No unchanged β-nicotyrine was detected in urine collected for 24 h following administration. In the same study, 15% of the administered nicotine dose was recovered. These data suggest β-nicotyrine is extensively metabolized in humans.

The metabolism of β-nicotyrine by rabbit liver and lung has been studied. NADPH-supplemented rabbit lung and liver microsomal preparations convert β-nicotyrine to four metabolites [48]. The primary metabolites (Figure 1-7) were shown to be an equilibrium mixture of two unstable pyrrolinone species, 1-methyl- 5-(3-pyridinyl)-4-pyrrolin-2-one (**4**) and 1-methyl-5-(3-pyridinyl)-3-pyrrolin-2-one (**5**). These isomeric pyrrolinones undergo autoxidation, to form 5-hydroxy-1-methyl-5-(3-pyridinyl)-3-pyrrolin-2-one (**6**), and hydration, to form 5'-hydroxycotinine (**7**)[49].

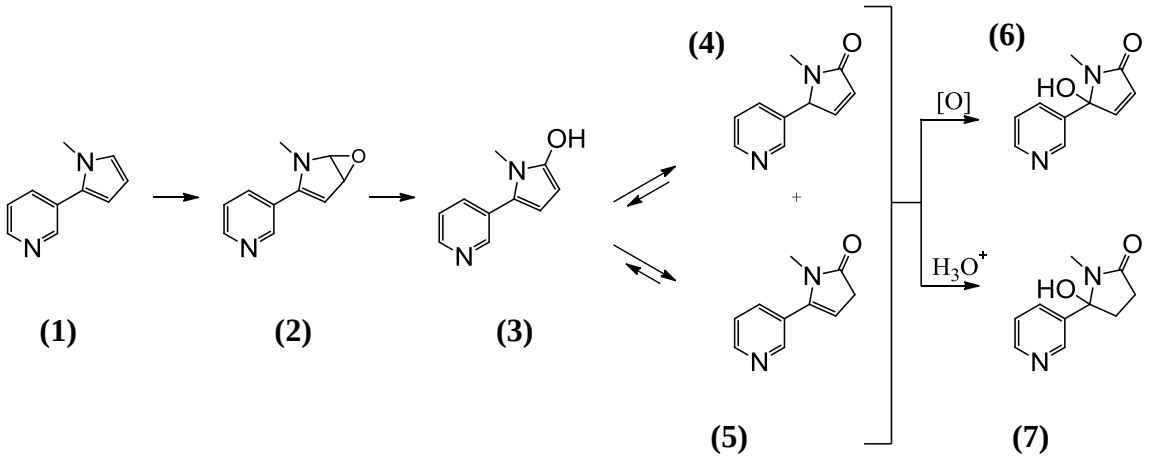


Figure 1-7. Proposed pathway for β-nicotyrine metabolism.

This thesis focuses on the interactions between tobacco-related molecules, such as β–nicotyrine or menthol, and the enzymes that are responsible for nicotine and NNK metabolism. Considering the large number of smokers and the highly addictive properties of tobacco, a better understanding of the molecular components of tobacco that may contribute to the development and maintenance of a tobacco addiction is warranted.

1.4 Cytochrome P450 Enzymes (P450s)

Nicotine and NNK are metabolized by enzymes that are members of the P450 superfamily. Cytochrome P450 enzymes comprise a large superfamily of monooxygenases that are present in animals, plants, fungi and bacteria. These enzymes were first discovered in 1958 as carbon-monoxide binding pigment in pig and rat liver microsomes [50;51] Later, the enzyme was characterized as a hemoprotein and called "P450" because of the Soret peak at 450 nm in their reduced form when saturated with carbon monoxide[52;53]. P450s contain an Fe(III) protoporphyrin-IX heme in their active site [54]. The absorption maximum at 450 nm depends on a properly incorporated heme. Nitrogen atoms of the porphyrin provide the first four ligands and a cysteine thiolate acts as the fifth ligand of the heme iron (Figure 1-8).

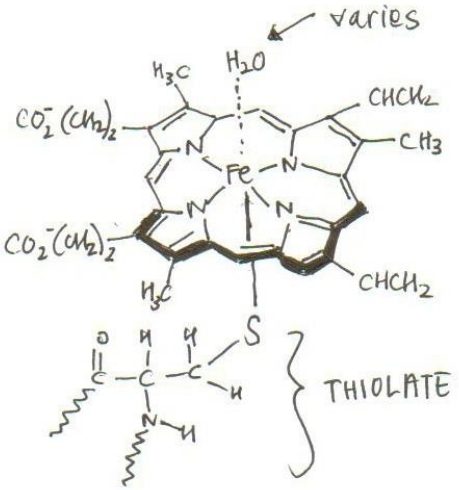


Figure 1-8. Fe(III) protoporphyrin-IX heme.

Carbon monoxide difference spectra are routinely used to quantify the amount of P450 enzyme that contains a properly incorporated heme. Substrate or inhibitor binding to P450s can be analyzed by following changes in the UV-visible heme Soret spectrum that result from displacement of the water or an amino acid ligand from the heme iron (type I spectrum), or from direct coordination of ligand to the heme iron (type II spectrum) [55].

P450s are capable of acting on a diverse range of endogenous and exogenous substrates. Reactions catalyzed by P450s include hydrocarbon hydroxylation, heteroatom oxidation and dealkylation (heteroatom release), aromatic ring oxidation, acetylene oxidation, dehydrogenation carbon-carbon bond cleavage and radical cross-linking of substrates [56;57]. Although there are several exceptions, the general reaction catalyzed by P450s is one of mixed-function oxidation (as shown below where S is the substrate and SO is the oxidized product). These reactions require molecular oxygen and reducing equivalents supplied by NADPH or NADH as well as a protein partner, P450 oxidoreductase, for electron transfer.

$$S + O_2 + 2\,e^- + 2\,H^+ \longrightarrow SO + H_2O$$

Most of the P450 catalytic cycle is widly accepted. The cycle starts with the heme iron in the ferric (Fe^{III}) state **(I)**. The substrate binds the low spin ferric heme iron, forming a high spin ferric iron **(II)**. Upon the addition of the first electron, which is supplied by NAD(P)H via redox protein partners, the iron is then reduced to a ferrous (Fe^{II}) state **(III)**. Ferrous heme iron then binds molecular oxygen (O_2) producing Fe^{II}-O_2 (not shown). This complex then undergoes a conversion to a more stable complex anion complex **(IV)**. Next, a second electron transfer, sometimes delivered from cytochrome *b*5, occurs. A comparison between the bond energies of O_2, O_2^- and O_2^{2-} suggest that the Fe^{III} O_2^{2-} complex is then formed **(V)**. Then, the first protonation occurs on the distal oxygen to generate an Fe^{III} hydroperoxo **(VI)**. A second distal protonation leads to

heterocyclic O-O bond scission, generating water and a porphyrin π cation radical iron V **(VII)**. Finally, the iron V species transfers its active oxygen atom to the substrate, forming monooxygenated product and regenerated ferric P450.

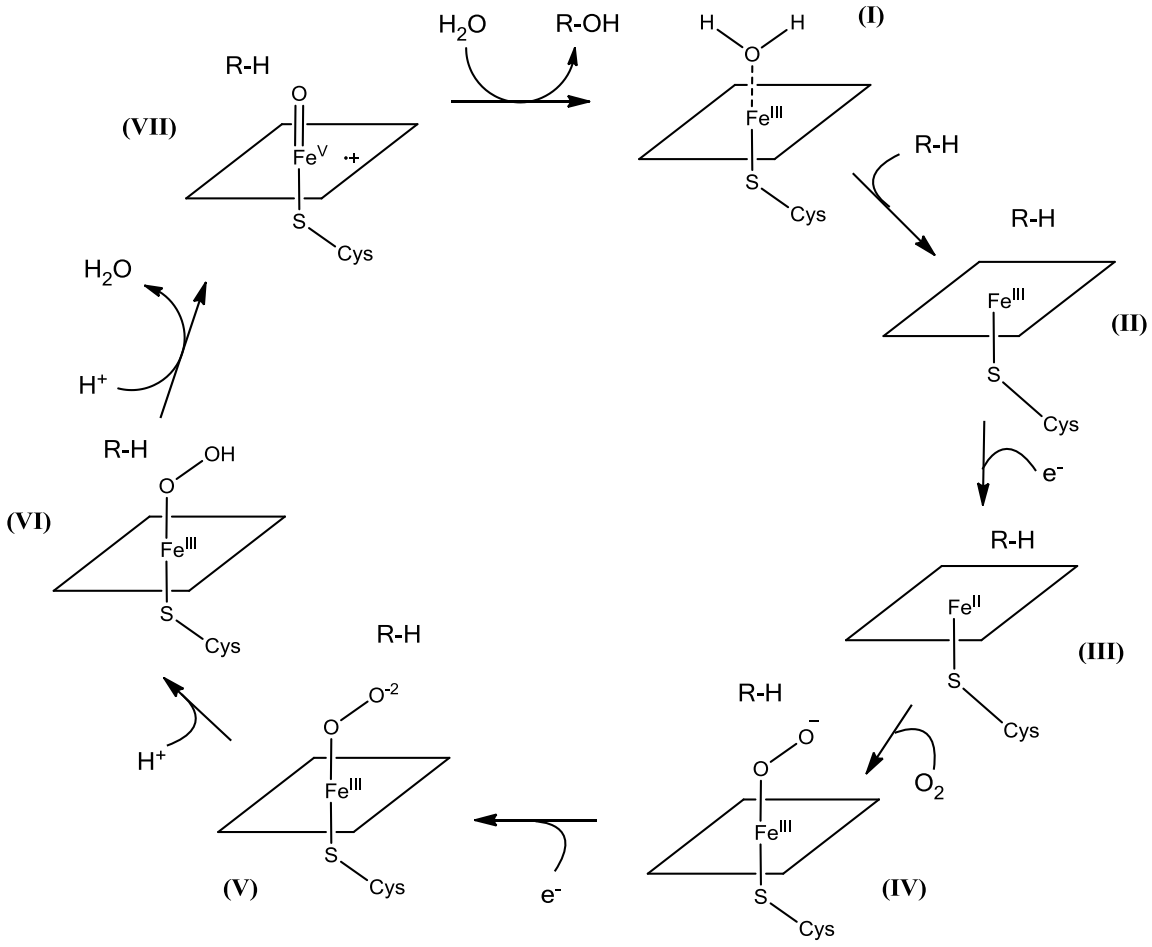


Figure 1-9. Cytochrome P450 catalytic cycle.

1.4.1 Human Cytochrome P450s

Human P450s contribute to normal development and homeostasis by metabolizing lipids, steroids, vitamins, eicosanoids, retinoids and prostaglandins. They also metabolize xenobiotics. There are 57 P450 genes as well as 58 pseudogenes that have evolved from several gene duplication and deletion events. Human P450s are membrane-bound and are primarily localized in the endoplasmic reticulum of the cells. Six P450s are found exclusively in the mitochondria; these are primarily involved in steroid metabolism [58].

There is wide variation in P450 activity. Regulatory networks including nuclear receptors and transcription factors define the tissue-specific distribution of individual P450s and modulate constitutive and inducible expression [59]. Many factors, both physiological and environmental, contribute to individual variability in P450 induction; included in this list are disease, gender, age, endocrine homeostasis, dietary components, and environmental ligands[60]. Xenobiotic-metabolizing P450s are highly polymorphic and this leads to variation in the levels of enzymes and catalytic function. Furthermore, P450 activity is affected by exposure to inhibitors. Over 90% of drugs on the market are metabolized by a relatively small number of P450s, 3A4/3A5>2D6>2C9>2E1≈1A2, listed here in order of their relative contribution to drug metabolism [61]. The role of these enzymes is extensively studied to predict the potential for adverse drug reactions and variation in drug efficacy.

1.4.2 Human P450 2A Subfamily

Two human P450 enzymes, P450 2A6 and P450 2A13, play key roles in the metabolism of nicotine and NNK and are thus of particular interest to those studying tobacco addiction and carcinogenesis and are the focus of this thesis [39;62-64]. P450 2A6 is primarily a hepatic enzyme and P450 2A13 is expressed in the respiratory tract [65;66]. Cytochrome P450 2A13 mRNA is highest in the respiratory tract, but only ~5- and ~9-fold higher than CYP2A6 mRNA in nasal mucosa and lung, respectively [65;67]. In contrast, in the liver CYP2A6 mRNA is ~1,900-fold higher than CYP2A13. In the liver, P450 2A6 is reported to make up 1-10% of the total P450 [68]. In human liver, xenobiotic metabolism by and inhibition of P450 2A13 is likely to be negligible, but in the respiratory tract, both P450 2A6 and P450 2A13 enzymes may contribute depending on the substrate or inhibitor.

P450 2A6 and P450 2A13 primary sequences are 94% sequence identical. Accordingly, overall the P450 2A13 and P450 2A6 structures are very similar (α-C of RMSD 0.5 A˚) [69]. The active site cavity for both enzymes is small and highly hydrophobic with a cluster of phenylalanine residues composing the active site roof. Amino acid differences between the two enzymes at positions 117, 300, 301, and 208 result in changed ligand orientations as well as a smaller active site volume in P450 2A6 (281.7 $A^{\circ 3}$) than P450 2A13 (309.4 $A^{\circ 3}$) [70]. These P450s have substantial overlapping substrate selectivity; substrates for these enzymes tend to be small molecules with planar rings, however there are exceptions, such as in the case of P450 2A13 metabolism of testosterone [71;72].

Despite the similarities, in some cases these two P450 2As exhibit different catalytic efficiencies and generate unique products [65;72-74]. One common substrate, coumarin, is frequently used to probe P450 2A activity. P450 2A6 selectively catalyzes the 7-hydroxylation of coumarin but, P450 2A13 catalyzes both the 7-hydroxylation and 3,4 epoxidation of coumarin [75]. Another marked difference in these enzymes is the superior ability of P450 2A13 to activate the tobacco-specific procarcinogen NNK through α-hydroxylation [65;72-74]. This hydroxylation results in a diazonium ion that can generate DNA adducts and initiate lung cancer [76]. P450 2A13 is a 200-fold more efficient catalyst of NNK hydroxylation (k_{cat}/K_m) than is P450 2A6 [39]. The high efficiency with which P450 2A13 activates NNK combined with its presence in respiratory tissues suggests that it plays a critical role in tobacco-induced lung cancer. P450 2A13 is also a somewhat superior catalyst of nicotine metabolism; catalyzing nicotine 5'-oxidation at a 5- to 20-fold higher catalytic efficiency than P450 2A6 [63;77].

1.4.3 Nicotine metabolism by P450 2As

Quantitatively, the most important metabolite of nicotine is the lactam derivative cotinine. In humans, typically 80% of nicotine is converted to cotinine [76]. Both P450 2A6 and P450 2A13 catalyze the 5'-oxidation of nicotine with higher catalytic efficiency than other P450s. The product of this 5'-oxidation generates the immediate product, 5'-hydroxynicotine, which is in equilibrium with the nicotine$^{\Delta 1',5'}$iminium ion (Figure 1-10). A second P450-catalyzed oxidation is then carried out converting the iminium ion to cotinine. *In vivo*, a second enzyme, aldehyde oxidase may convert the iminium ion to

cotinine. In humans, due to its presence in the liver, P450 2A6 is the main catalyst of nicotine 5'-oxidation [26]. Individuals with no P450 2A6 due to a genetic deletion have a greater than 85% reduction in 5'-oxidation [78-80].

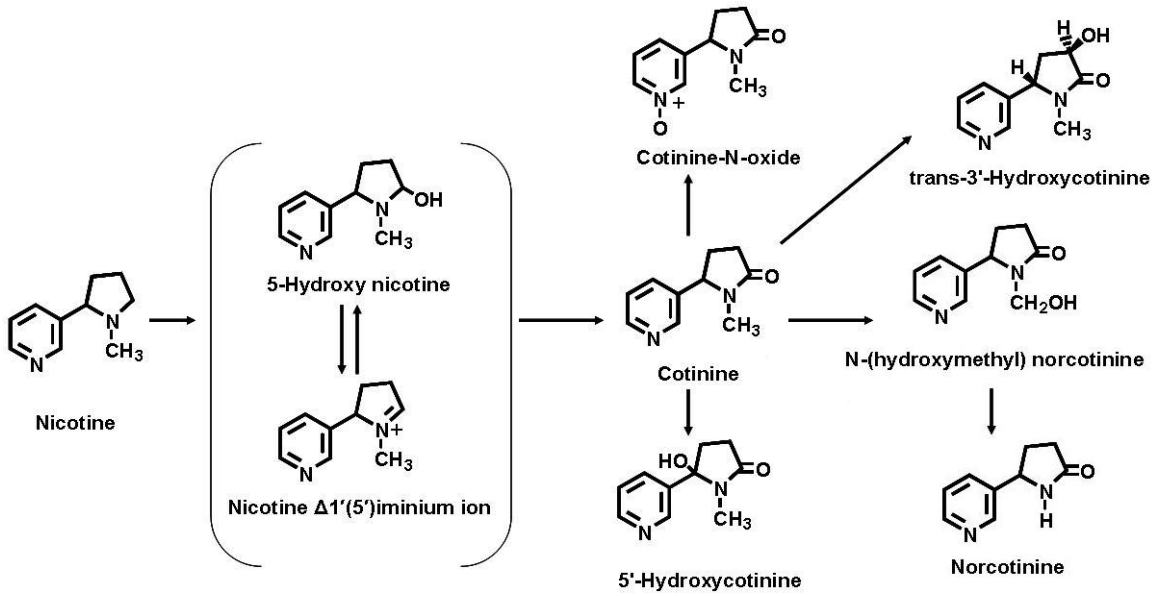


Figure 1-10. Major pathways of nicotine metabolism in humans.

Both in vitro and in vivo studies have demonstrated considerable interindividual variation in P450 2A6 activity. [81-84] Genetic variation in the P450 2A6 gene locus has been shown to contribute to this variability. To date, 38 numbered and 2 duplication alleles have been identified[85]; nineteen of these have been shown to have reduced activity *in vitro* and or *in vivo.* In some studies, individuals who are heterozygous for defective alleles smoked fewer cigarettes per week than smokers homozygous for wild-type and were less likely to become nicotine dependent [86;87]. This genetic variation affecting P450 2A6 activity is not generally associated with adverse effects on drug

clearance, suggesting that P450 2A6 inhibition is unlikely to alter the metabolism of other drugs [58].

Pharmacologically blocking P450 2A6 enzymatic activity decreases the rate of nicotine elimination and subsequently can reduce the extent of smoking [88;89]. Therefore, the identification and characterization of specific and potent inhibitors of P450 2A6 will contribute to the design of drugs to modify tobacco consumption.

Mechanism based inactivation, or suicide inhibition, of P450 2A6 and P450 2A13 has been reported in a number of studies, but is fully characterized in very few. Mechanism-based inactivators are specific and potent inhibitors and harnessing the relevant chemistry would lead to powerful drug design. Inactivation of both P450 2A6 and P450 2A13 occurs during nicotine metabolism [77]. However, nicotine is not the agent of inactivation; a secondary or even tertiary nicotine metabolite is likely the inactivator. The species responsible for enzyme inactivation has yet to be identified, however one possible contender is β-nicotyrine. It has been suggested that β-nicotyrine is a mechanism-based inactivator of P450 2A6, however, the data supporting this is minimal[90].

1.4.4 β-nicotyrine as a potential metabolite of nicotine

Nicotine metabolism has been extensively characterized in humans and 90% of a systemic dose of nicotine can be accounted for as nicotine and metabolites in the urine [35;91]. It is possible that β-nicotyrine could be among metabolites that make up the remaining ten percent. To date, two literature references suggest β-nicotyrine could be a

metabolite of nicotine [92-94]. In dogs and rats, he first identified that β-nicotyrine is a urinary metabolite of (S)-nicotine. The second was an *in vitro* experiment with liver homogenate from rabbit and guinea pig in which β-nicotyrine was detected as an oxygen and NADPH-dependent metabolite of nicotine. This shows that, in rodents and dogs, β-nicotyrine is a product of nicotine metabolism. β-Nicotyrine has not been identified as a nicotine metabolite in humans. Regardless, smokers are exposed to β-nicotyrine since it is in tobacco and therefore an understanding of the impact of this compound on nicotine metabolism is warranted.

1.5 Goals of this research

The importance of understanding the effects of tobacco constituents on P450 2A6 and P450 2A13 activity is underscored by their potential impact on smoking behavior and NNK activation. In addition, the characterization of potential mechanism-based inactivators may aid in the design of potent and specific inactivators of these enzymes. That could lead to drug development to aid in smoking cessation.

Chapter 2

Substrate binding and inhibition of Cytochrome P450 2A6 and Cytochrome P450 2A13

2.1 Introduction

Tobacco use results in exposure to a number of harmful compounds and subsequently can lead to a multitude of negative health outcomes, including cancer. Included in the list of harmful compounds is the tobacco specific carcinogen NNK, which forms during the curing of tobacco. Initiation of carcinogenesis by NNK occurs as a result of metabolic activation by P450-catalyzed α–hydroxylation to a reactive species that alkylates DNA [39].

Discontinued use of tobacco products lowers the risk of developing lung and other types of cancer. Despite the potential health consequences, many smokers are unable to quit tobacco use due to the presence of the highly addictive compound, nicotine. Nicotine is not a carcinogen and toxicity due to nicotine exposure from cigarettes is rare; however nicotine metabolism is of interest with regard to understanding nicotine addiction and smoking cessation. Decreased rate of nicotine metabolism may modify smoking behavior [95] and pharmacologically blocking nicotine metabolism has been reported to reduce the extent of smoking [88].

The human P450 enzymes responsible for the metabolism of nicotine and the activation of NNK are P450 2A6 and P450 2A13. Although both enzymes catalyze nicotine metabolism, P450 2A6-mediated oxidation of nicotine is the primary route of elimination due to its high concentrations of in the liver [26]. Pathways key to NNK activation are P450 2A13 catalyzed hydroxylations [39]. Both P450 2A6 and P450 2A13 catalyze these reactions, however structural differences between the two enzymes render P450 2A13 the superior catalyst of this reaction [69;96].

Although P450 2A6 and P450 2A13 enzymes share 94% sequence identity and nearly identical secondary structure, there is marked structural variation in the active sites of these two enzymes. Five of the 32 amino acid differences between P450 2A6 and P450 2A13 are located in the active sites. These differences lead to a 15-20% increase in the volume of the P450 2A13 active site compared to P450 2A6 (250 $Å^3$ and 307 $Å^3$, respectively). In addition, the active site geometry of P450 2A6 and P450 2A13 are dissimilar. The geometry directs ligand binding to the most favorable orientation. This sometimes results in a ligand having a different orientation in the P450 2A6 active site than it does in P450 2A13 [69;70]. Modeling studies have predicted that NNK binding in the P450 2A13 active site sits in a favorable orientation for the generation of a carcinogenic metabolite, whereas in P450 2A6 it does not.

Due to their roles in the metabolism of nicotine and NNK, inhibition of P450 2A6 and P450 2A13 may modify tobacco consumption and mitigate carcinogenesis. Therefore, compounds found in tobacco that potentially inhibit these enzymes are of

interest. In this study, two of these compounds, menthol and β-nicotyrine are investigated.

Menthol is a tobacco additive that has become the focus of recent FDA regulation. Epidemiological studies have reported smokers of mentholated versus non-mentholated cigarettes have an increased risk of cancer [97-99]. More recently, however, a large and well-designed study found that lung cancer incidence and mortality actually decreased among older adult smokers of mentholated cigarettes compared to smokers of non-mentholated cigarettes [100]. Regardless, a report that menthol inhibits nicotine metabolism has prompted further study on the mechanistic basis of this outcome [15].

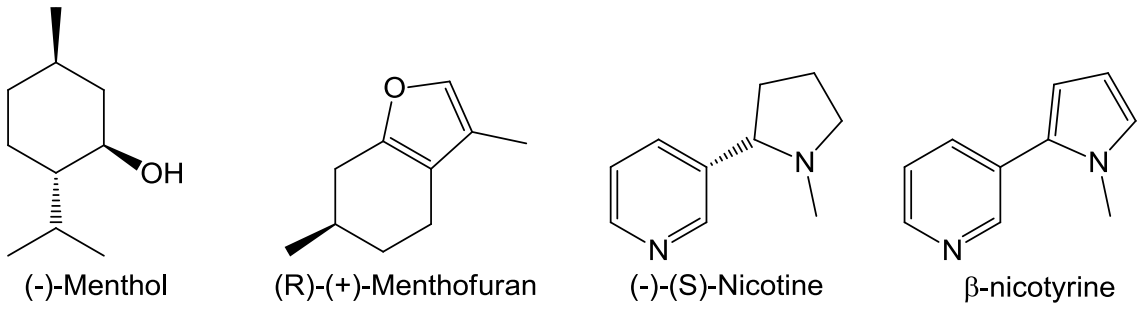


Figure 2-1. Structure of menthol, menthofuran, nicotine and β-nicotyrine.

β-nicotyrine is the pyrrolic analogue of nicotine (Figure 2-1). It is reported to be present in tobacco at low levels (4-40 μg/cigarette)[42]. In rats, administration of β-nicotyrine decreased the rate of nicotine metabolism and *in vitro* it inhibits P450 2A6 [90;101]. Additionally, it was suggested that β-nicotyrine is a mechanism-based inactivator (MBI) of P450 2A6 [90]. Based on the little that is known regarding the level of human

exposure to β-nicotyrine, its effect, if any, on nicotine and NNK metabolism remain unclear.

The use of MBIs, also known as suicide inhibitors, are attractive candidates in the design of new drugs [102;103]. The broad definition of an MBI includes any compound that is catalytically converted to a form that irreversibly inactivates the enzyme carrying out the enzymatic conversion [104]. MBIs can be used in the design of drugs that, because of their catalytic requirement, selectively inhibit the target enzyme. Additionally, since inactivation is irreversible, the potency of a drug that acts as an MBI will be dependent on the rate of synthesis of that particular enzyme.

Mechanism-based inactivation of P450 2A6 and P450 2A13 has been reported for several compounds, however it is well characterized for only a few; among these are menthofuran and 8-methoxypsoralen (8-MOP). Menthofuran has been identified as a potent MBI of P450 2A6. Menthofuran-mediated loss of P450 2A6 activity was dependent on catalytic turnover and immunohistochemistry analysis provided evidence that the apo-protein was modified [105]. 8-MOP, a MBI of human P450 2A6, has been shown to inhibit the conversion of nicotine to cotinine both in vitro and in vivo [106;107]. Inactivation of both P450 2A6 and P450 2A13 occurs during nicotine metabolism [77]. Nicotine, however, is not the agent of inactivation; a secondary or even tertiary nicotine metabolite is likely the inactivator. The species responsible for enzyme inactivation has yet to be identified; one possible contender is β-nicotyrine. β-Nicotyrine is a urinary metabolite of nicotine in dogs and rats[108]. Therefore, in addition to direct exposure of

smokers to β-nicotyrine in tobacco, exposure to this potential MBI may occur through nicotine metabolism. It has not yet been identified as a nicotine metabolite in humans

The importance of understanding the effects of tobacco constituents on P450 2A6 and P450 2A13 activity is underscored by their potential impact on smoking behavior and NNK activation. In addition, the characterization of potential mechanism-based inactivators may aid in the design of specific inactivators of these enzymes. The objectives of the current study were to determine the relative inhibition of P450 2A6 and P450 2A13 activity by β-nicotyrine, menthol and menthofuran and to assess inactivation of P450 2A6 and P450 2A13 by each of these compounds.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

Menthofuran, menthol, 7-hydroxycoumarin, coumarin, dilauroyl- L-α-phosphatidylcholine (DLPC), NADPH, bovine serum albumin, catalase, and all other biochemical reagents were obtained from Sigma–Aldrich (St. Louis, MO) and were of analytical grade. β-Nicotyrine (99% pure) was obtained from Toronto Research Chemicals. Trifluoroacetic acid (TFA) was obtained from Pierce Chemical (Rockford, IL).

2.2.2 Protein Expression and Purification

The enzymes used in this study were heterologously expressed in *Escherichia coli* and purified according to previously published methods [71;109;110]. His-tagged P450 2A13 and P450 2A6 are full-length enzymes. At saturating concentrations of coumarin (40 or 100 µM) the rates of coumarin 7-hydroxylation for both P450 2A13 and P450 2A6 were comparable with those reported previously [111].

2.2.3 Reconstitution of Enzymes

P450 2A6 and P450 2A13 were reconstituted with rat NADPH-P450 oxidoreductase (reductase) in a 1:2 ratio with lipid (DLPC, 0.2 µg/pmol P450) and incubated for 45 min at 4 °C. Then, 50 mM Tris buffer, pH 7.4 and catalase were added to give final concentrations of 1 pmol/µl P450 2A, 2 pmol/µl reductase, 0.2 µg/µl lipid, and 60 U/µl catalase.

2.2.4 Spectral binding assays

Binding titrations with the ligands were conducted at 20°C using a DW2 UV/vis spectrophotometer (OLIS, Bogart, GA). P450 was diluted to 1 µM in 100 mM potassium phosphate buffer, pH 7.4. Diluted enzyme was divided equally between two 1.0-ml quartz cuvettes (1-cm path length), and a baseline was recorded (300–500 nm). Freshly prepared aliquots of ligand dissolved in 10% ethanol were added to the sample cuvette. Concentrations ranged from 1nM to 400µM. An equal volume of 10% ethanol was

added to the reference cuvette. Difference spectra were collected (300–500 nm) after being allowed to equilibrate at RT for 5 minutes. During the spectral titrations, the total amount of ethanol added did not exceed 2%. Binding to P450 was monitored as the absorbance difference (ΔA) between the minimum (420 nm) and the maximum (385 nm). The apparent binding constant (K_D) and the maximum spectral change (A_{max}) were determined from nonlinear least-squares regression using Sigma Plot kinetics program

FromSystat Software Inc. (Chicago, IL).

2.2.5 P450 coumarin 7-hydroxylation activity

The reaction mixtures contained reconstituted enzyme (5 pmol P450), coumarin (0.4–20 µM), NADPH-generating system (0.4 mM NADP, 10 mM glucose 6-phosphate, and 0.4 units/ml glucose phosphate dehydrogenase) and 12µg bovine serum albumin in 300 µl 50 mM Tris buffer, pH 7.4. The reaction was allowed to proceed for 10 min at 37°C prior to termination by the addition of 30 µl of 15% trichloroacetic acid. To investigate inhibition, P450 2A6 or P450 2A13 activity was measured in the presence of menthofuran, menthol or β-nicotyrine. Experiments were carried out within the linear range of product formation. P450 2A6 reactions contained 0, 1, 2 and 3 µM menthofuran, 0, 1, 3 and 5 µM β-nicotyrine, or 0, 50, 100 and 200 µM menthol. P450 2A13 reactions contained 0, 5, 10 and 25 µM menthofuran, 0, 1, 5 and 10 µM β-nicotyrine, or 0, 50 100 and 200 µM menthol. 7-Hydroxycoumarin was quantified by HPLC with fluorescence detection [111]. K_m, V_{max} and K_I values were determined using the Sigma Plot kinetics program from Systat Software Inc. (Chicago, IL). K_i estimates were determined using

nonlinear regression analysis. All data were fit to a competitive inhibition model (Eq. (1)) and tested using the Runs test of residuals to determine statistically whether experimental data are randomly distributed around the curve with 95% confidence.

$$V_0 = V_{max}[S] / (K_m(1 + [I]/K_i) + [S])$$ **Equation 1**

Additionally, global R^2 values to assess the goodness of fit confirmed that the competitive model fit the data sets well ($R^2 = 0.92$) except for the data set from menthofuran inhibition of P450 2A13 ($R^2 = 0.84$). All data sets passed the Runs test except for the data set from β-nicotyrine inhibition of P450 2A6.

2.2.6 Inactivation

Inactivation was studied in three types of experiments. Consistent in all three, primary reaction mixtures containing menthofuran, menthol or β-nicotyrine and the reconstituted enzyme mixture described in Section 2.2.3 were pre-incubated for 5 min at 30 °C prior to the addition of 1 mM NADPH (or water as a control). At various times (including 0 minutes as a control), aliquots (5 µl) were removed and added to 300 µL of a secondary reaction mixture (20 µM coumarin, the NADPH-generating system and 40 µg/ml bovine serum albumin in 50 mM Tris buffer, pH 7.4) and incubated for 10 min at 30 °C, then 7-hydroxycoumarin formation was quantified as described in Section 2.2.5. Inactivation experiments were carried out at 30°C in order to minimize the loss of P450

2A6 activity in the presence of NADPH and no inactivator and to be comparable to previous literature [77;111].

The first inactivation experiment compared menthofuran, menthol or β-nicotyrine as potential inactivators. Primary reaction mixtures contained uniform inhibitor concentration (20 µM) and were allowed to incubate for 10 minutes. For inhibitor and P450 combinations that showed no evidence of inactivation, subsequent experiments were performed using increased inhibitor concentrations (200 µM) and time (30 min). After the indicated amount of time, aliquots were removed and added to the secondary reactions.

The second type of experiment determined the kinetic parameters for the menthofuran and β-nicotyrine-mediated inactivation of P450 2A6. Either menthofuran (0–10 µM) or β-nicotyrine (0–80 µM) were incubated with the primary reaction mixture for the indicated times (0–5 min), after which aliquots (5 µl, 5 pmol P450) were removed and added to the secondary reaction mixture (as described in the first paragraph of this section). Kinetic rate constants were determined from the slopes of the lines when the logarithm of the percent activity remaining was plotted against time.

The third type of inactivation experiment determined the partition ratio for menthofuran and β-nicotyrine-mediated inactivation of P450 2A6. The primary reaction mixtures containing menthofuran (0–100 µM) or β-nicotyrine (0–100 µM) were incubated for 0 or 30 minutes before aliquots were removed and added to the secondary reaction mixture. The partition ratio was estimated from the intercept of the regression

line obtained at low [inhibitor]/[2A6] ratios and the line obtained at saturating inhibitor concentrations [112].

2.3. Results

2.3.1 Ligand binding

Four compounds (Figure 2-1) were characterized for their binding mode and affinity for P450 2A6 and P450 2A13 (Table 2-1). Difference spectra collected upon ligand titration of both enzymes with all four compounds resulted in increased absorbance at 379-387 nm and decreased absorbance at 414-420 nm (Fig 2-2). These changes are typical of type I interactions, which are typical of substrate binding. Type I binding occurs when a ligand displaces the 6^{th} iron ligand, typically water, resulting in a penta-coordinated heme. None of the compounds displayed type II binding, which is typical of an inhibitor. Type II binding is indicated by an increased absorbance at 431-432 nm and decreased absorbance at 406-412 nm as a result of replacement of the water ligand with the compound, maintaining the hexa-coordinated heme iron.

The equilibrium dissociation constants were determined for P450 2A6 and P450 2A13 for each compound. K_d values ranged from 1 to 85μM for P450 2A13 and 5 to 103 μM for P450 2A6 (Table 2-1). The compounds with the highest affinity for both P450 2A13 and P450 2A6 were menthofuran (K_d, 1 μM and 5 μM, respectively) and β-nicotyrine (2 μM and 7 μM, respectively). Nicotine had an intermediate affinity for P450 2A13 and P450 2A6, (46 μM and 24 μM, respectively) leaving menthol with the lowest affinity for these enzymes (85 μM and 103 μM, respectively).

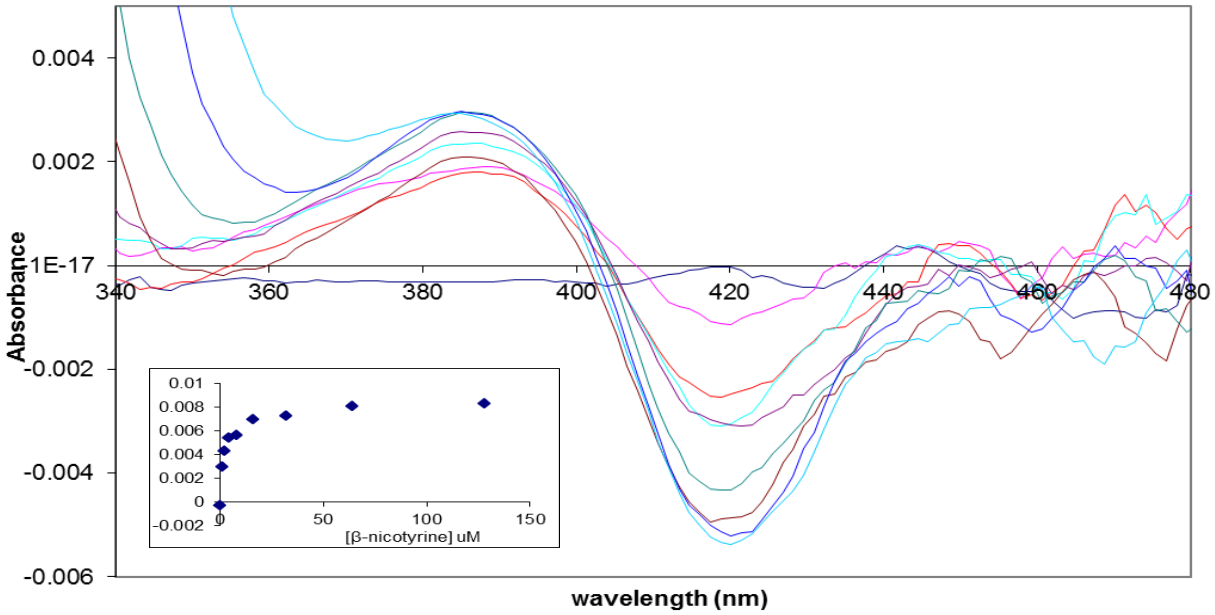


Fig 2-2. UV-visible difference spectra from titration of P450 2A13 with increasing concentrations of β-nicotyrine. Not all spectra are shown. Inset is the nonlinear regression analysis completed using Sigma Plot from which the K_D is obtained.

Table 2-1. Calculated dissociation constants for P450 2A binding to menthofuran, β-nicotyrine and menthol and nicotine.

Ligand [a]	K_D(μM) [b]	
	P450 2A6	P450 2A13
Menthofuran	5 ± 1.2	1 ± 0.13
Menthol	103 ± 9.3	85 ± 1.7
Nicotine	24 ± 2.3	46 ± 0.86
β-nicotyrine	7 ± 2.1	2 ± 0.96

[a] Samples were prepared as described in the Materials and methods.

[b] K_D is obtained by nonlinear regression analysis completed using Sigma Plot. Values are means ± S.D. from three experiments.

2.3.2 Inhibition

The relative inhibition of P450 2A6 and P450 2A13-catalyzed coumarin 7-hydroxylation by menthol, β-nicotyrine and menthofuran were determined and the kinetic constants are presented in Table 2-2. As previously reported, the K_m of P450 2A13-catalyzed coumarin 7-hydroxylation was lower than the K_m for P450 2A6 (3.7 and 6.7 μM, respectively)[75;111]. Both menthofuran and β-nicotyrine were relatively potent inhibitors of P450 2A6 activity, with K_I values of 0.29 μM and 1.07 μM, respectively. Inhibition of P450 2A6 by menthol was 100 times less potent (K_I of 110 μM). The K_I for menthol inhibition of P450 2A13, 8.17 μM, was 13 times less than that of P450 2A6. β-Nicotyrine was also a more potent inhibitor of P450 2A13 than of P450 2A6. The inhibition data were analyzed according to several kinetic inhibition models; however, no model was determined to be a superior fit to the data as indicated by the goodness of fit (R^2) values. The reported K_I values were determined using the competitive inhibition model and should be considered the apparent K_I.

Table 2-2. Calculated inhibition constants for inhibition of P450 2A activity by menthofuran, β-nicotyrine and menthol[a]

Inhibitor	K_I (μM)	
	P450 2A6	P450 2A13
Menthofuran	0.63 ± 0.07	1.17 ± 0.16
β-nicotyrine	1.07 ± 0.02	0.17 ± 0.003
Menthol	109.5 ± 10.7	8.17 ± 0.86

[a] Values are means ± S.D. from three experiments carried out in duplicate. The inhibition of CYP2A6 and CYP2A13-mediated coumarin 7-hydroxylation was determined as described in the materials and methods. Curves were generated using non-linear regression analysis.

2.3.3 Inactivation

To compare the relative potential of menthofuran, β-nicotyrine or menthol to inactivate either P450 2A6 or P450 2A13, an experiment was carried out at a single inhibitor concentration (20 μM). Incubation of P450 2A6 in the presence of NADPH with either menthofuran or β-nicotyrine resulted in the loss of coumarin 7-hydroxylation activity. Menthofuran was a particularly potent inactivator; only 5% activity remained after ten minutes. β-Nicotyrine inactivation was more modest with 49% activity remaining after ten minutes (Table 2-3). In contrast to the results with P450 2A6, no significant loss of P450 2A13 activity was observed with either menthofuran or β-nicotyrine. Neither increasing the concentrations nor incubation time resulted in any significant inactivation of P450 2A13. Using concentrations of 100 μM menthofuran or 200 μM β-nicotyrine and incubation a time of 30 min, no loss of P450 2A13-catalyzed coumarin 7-hydroxylation was detected (data not shown). Therefore, menthofuran and β-nicotyrine-mediated inactivation of P450 2A13 does not occur or it is too minor to detect. There was no indication that menthol inactivated either P450 2A6 or P450 2A13 (Table 2-3).

Table 2-3. Loss of P450 2A activity following incubation with menthofuran, β-nicotyrine or menthol[a]

Inhibitor (20 μM)	% Activity Remaining[b]	
	P450 2A6	P450 2A13
Menthofuran	5 ± 1	91 ± 5
β-nicotyrine	49 ± 10	87 ± 2
Menthol	97 ± 6	87 ± 11

[a] Samples were prepared as described in the Materials and methods. Reconstituted enzyme was incubated for 10 minutes at 30 °C in the presence of 20 μM Menthofuran, β-nicotyrine or menthol.

[b] Values are means ± S.D. from three experiments. Percent activity remaining is compared to control samples that contained no NADPH.

Inactivation of P450 2A6 by menthofuran and β-nicotyrine was not reversible upon removal of unbound small molecules. In three experiments, the P450 2A6-catalyzed coumarin 7-hydroxylation activity that remained following menthofuran inactivation (8 ± 5% relative to a minus NADPH control) was unchanged after the sample was passed through a size exclusion column (5 ± 3%). Similarly, the coumarin 7-hydroxylation activity of β-nicotyrine inactivated P450 2A6 (34 ± 2%) was not recovered when the sample was filtered through the column (29 ± 3%).

To assess the potential modification of the heme and apoprotein after inactivation of P450 2A6 by menthofuran or β-nicotyrine, two experiments were carried out. In the first, inactivated P450 2A6 was quantified by a reduced CO difference spectra and the loss in spectrally active enzyme was compared to the loss of 7-hydroxycoumarin activity relative to a non-NADPH-treated control. After menthofuran treatment, the 7-hydroxycoumarin activity remaining was 28.5 ± 1.6%, comparable to the amount of spectrally active P450 2A6 enzyme remaining 36 ± 4.6% (Table 2-4). Likewise, after β-nicotyrine treatment the enzyme activity remaining was the same as the amount of spectrally active P450 2A6 remaining (Table 2-4). The second experiment quantified the amount of native heme by HPLC analysis. For both compounds, the loss in activity after incubation in the presence of NADPH was not accompanied by a loss in native heme, and only one peak was observed, indicating no modified heme was present (Figure 2-3). These results together with the observed loss in the spectrally quantifiable protein, suggest that the inactivation of P450 2A6 by both menthofuran and β-nicotyrine was due to modification of the apo-protein.

The kinetic parameters of menthofuran and β-nicotyrine-mediated P450 2A6 inactivation were determined (Fig 2-4). The loss in P450 2A6 activity was dependent on the concentration of menthofuran or β-nicotyrine and increased with time. The K_I for the inactivation of P450 2A6 by menthofuran, 2.2 μM, was almost 50-fold lower than that of β-nicotyrine, 106 mM. The maximum rate constants for menthofuran- and β-nicotyrine-mediated inactivation, k_{inact}, were 1 and 0.61 min^{-1}, respectively; the $t_{1/2}$ values were 0.7 and 1.1 min, respectively.

The partition ratio, the number of moles of product formed per mole of enzyme inactivated, was determined using the titration method [104]. In brief, after complete inactivation of P450 2A6 with various concentrations of menthofuran or β-nicotyrine, the percentage of remaining P450 2A6 activity at each substrate concentration was plotted against the molar ratio of compound to enzyme (Figure 2-5). The intercept between the linear regression line obtained at low molar ratios and the horizontal line obtained from saturating conditions were extrapolated to the x-axis to yield the partition ratio. The partition ratio was more than threefold lower for β-nicotyrine compared to menthofuran (10 and 33, respectively).

Table 2-4. Relationship of P450 2A6 inactivation to spectrally active enzyme levels[a]

Inactivator	Activity Remaining	Spectrally active P450
Menthofuran	28.5±1.6[b]	36±4.6[b]
β-nicotyrine	55.5±0.7[c]	48±7.3[c]

[a] Reconstituted enzyme was incubated for 10 minutes at 30 °C in the presence of 20 μM menthofuran or 50 μM β-nicotyrine. Control samples contained no NADPH. The samples were analyzed for the coumarin 7-hydroxylation activity and the spectrally active enzyme quantified by reduced CO spectra. Values are means ± S.D. of percent coumarin 7-hydroxylation activity and percent spectrally active CYP2A6 remaining from three experiments.

[b] Values were not significantly different, unpaired T-test p=0.068.

[c] Values were not significantly different, unpaired T-test p=0.15.

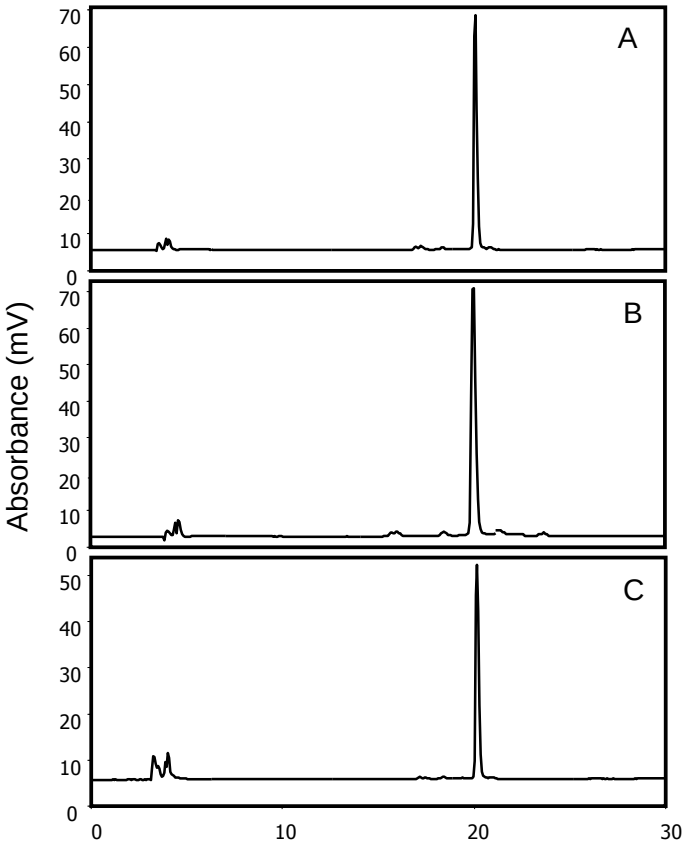


Figure 2-3. HPLC/UV chromatograms of P450 2A6 reactions. Reconstituted P450 2A6 was incubated for 10 minutes at 30 °C in the presence of 20 μM menthofuran (A), β-nicotyrine (B) or control (no inhibitor) and analyzed by LC/UV-vis at 405 nm. Heme elutes at 20 minutes.

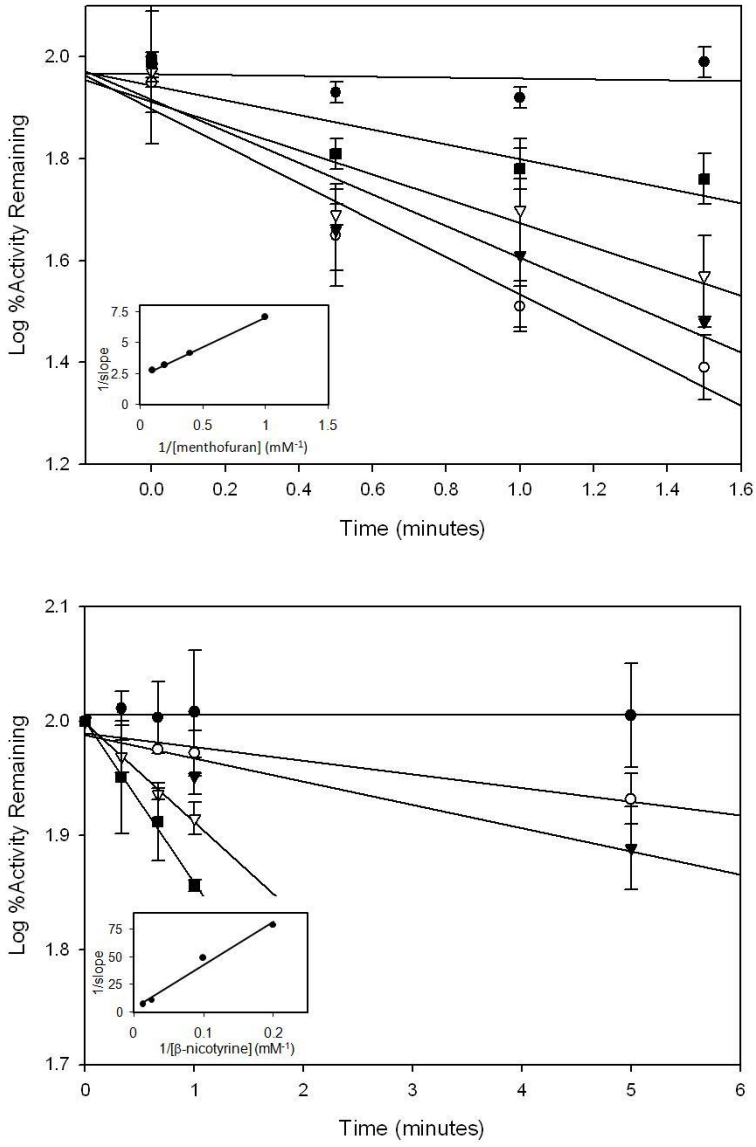


Figure 2-4. Time and concentration-dependent inactivation of P450 2A6 by menthofuran (A) and β-nicotyrine (B). Activity remaining refers to coumarin 7-hydroxylation activity determined in a secondary reaction. The β-nicotyrine concentrations used were 0(●), 5(■), 10(▽), 40(▼) and 80(○) μM and the menthofuran concentrations used were 0(●), 1(○), 2.5(▼), 5(▽) and 10(■) μM. Values are the mean ± S.D. from three independent experiments. The insets represent the double-reciprocal plot generated from the slopes of the lines at the various concentrations.

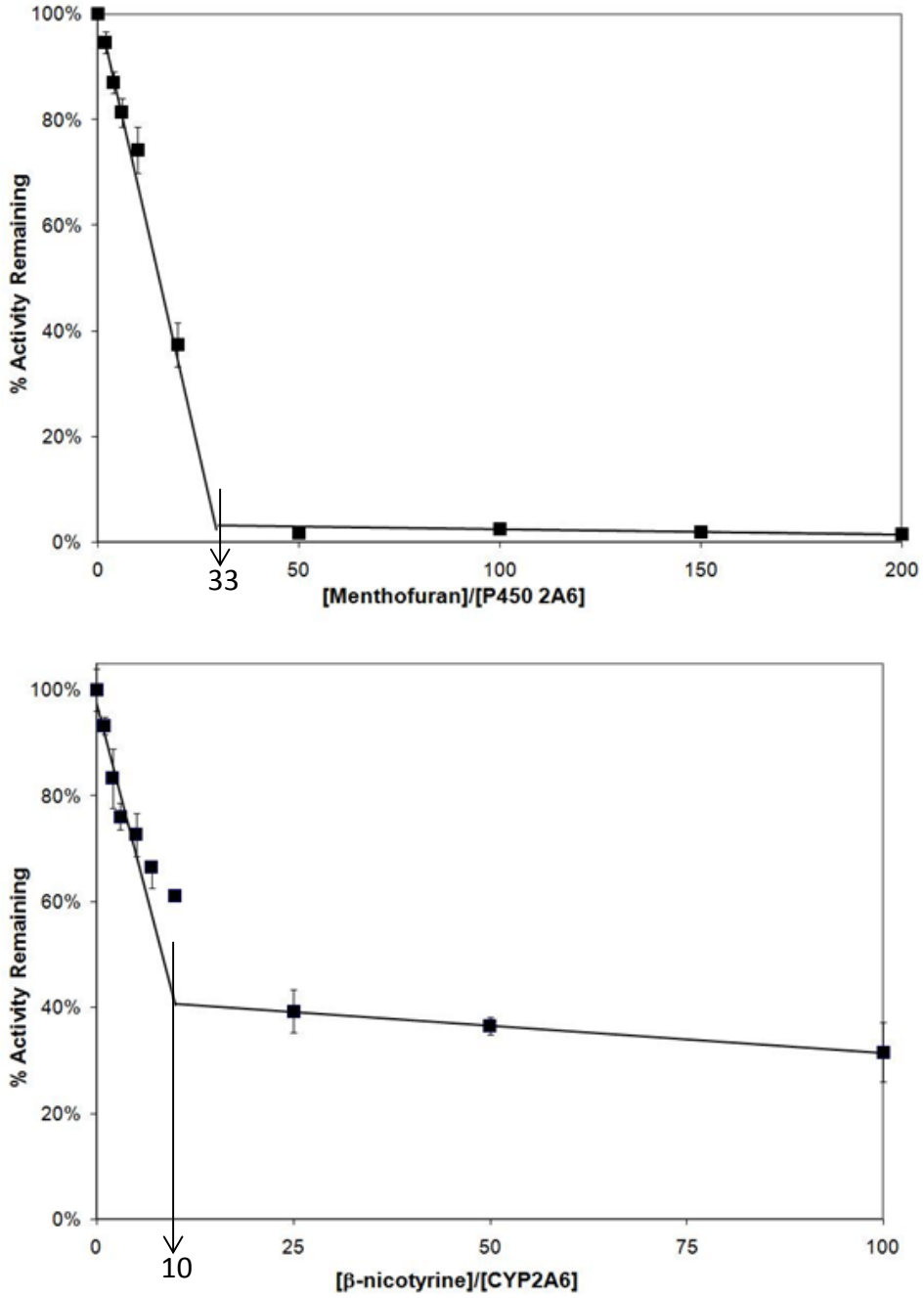


Figure 2-5. Partition ratio determination for CYP2A6 with β-nicotyrine (A) and menthofuran (B). The inactivation was allowed to go to completion, and then coumarin 7-hydroxylation activity was determined in a secondary reaction as described under *Materials and Methods*. Values are the mean ± S.D. of three independent experiments performed in duplicate. The arrow indicates the intercept used to determine the partition ratio.

2.4 Discussion

P450 2A6 and P450 2A13 are critical catalysts of nicotine and NNK metabolism. It is therefore relevant to characterize potential inhibitors of these enzymes as they may aid in smoking cessation and mitigate tobacco-specific carcinogenesis. In the study presented here, the inhibition and inactivation of P450 2A6 and P450 2A13 by the tobacco constituents, β-nicotyrine and menthol, were characterized and compared to the potent P450 2A6 inactivator, menthofuran. Both compounds inhibited the coumarin 7-hydroxylation activity of these enzymes. Yet, despite the high degree of similarity between P450 2A6 and P450 2A13, the effect of menthol, β-nicotyrine and menthofuran on each of these enzymes were quite different. Menthol did not inactivate either enzyme. It was a significantly better inhibitor of P450 2A13 than P450 2A6 and as indicated by the lower K_d, P450 2A13 bound menthol more tightly than P450 2A6. β-nicotyrine was also a more potent inhibitor of P450 2A13 than P450 2A6, and the K_d of P450 2A13 for β–nicotyrine was lower than for P450 2A6. In addition, β-nicotyrine was an MBI of P450 2A6 but not P450 2A13. The well-characterized MBI of P450 2A6, menthofuran, was also not an inactivator of P450 2A13. This observed enzyme specific inactivation is consistent with the previously reported unique substrate specificity for these enzymes, and is likely due to the larger active site and more permissive geometry of P450 2A13 compared to P450 2A6 [69].

Menthol cigarettes are used by the majority of African American smokers (85%), but only 10–20% of European Americans (NSDUH report). The prevalence of menthol cigarettes among African American smokers was hypothesized to contribute to their

higher incidence rate of lung cancer compared to European Americans [97;98;113]. However, the proposed link of menthol cigarette use to lung cancer was strongly refuted by the results of a recent prospective epidemiology study [100]. The conclusion of that study was that menthol cigarettes are potentially less harmful, not more, than non-menthol cigarettes. Part of what was driving the original hypothesis was the observation that the use of menthol cigarettes affected cigarette smoking behavior and P450 2A6-mediated nicotine metabolism [15]. In the study reported here, menthol was found to be a poor inhibitor of P450 2A6 and did not inactivate the enzyme. MacDougall et al. also reported that menthol is a weak inhibitor of P450 2A6 [16]. These results suggest that the presence of menthol will have little, if any, effect on the rate of nicotine metabolism in humans. Supporting this conclusion, Williams et al. found no evidence of modified nicotine intake when smokers used menthol cigarettes [114].

The effect of menthol on P450 2A13 has not previously been reported. Here we report that menthol is a modest inhibitor of P450 2A13. Inhibition of P450 2A13 by menthol, which is present in tobacco smoke at much higher levels than NNK, might mitigate NNK activation by P450 2A13 in the lung. This inhibition could contribute to the results observed by Blot et al showing that the overall odds ratio of lung cancer is significantly lower among menthol compared with non-menthol smokers [100].

β-Nicotyrine, a minor tobacco alkaloid, has previously been reported to inhibit nicotine metabolism in rodents. The pretreatment of mice with β-nicotyrine resulted in a significant increase in tissue and blood levels of nicotine[101]. In addition, there is evidence in rabbit lung cells and microsomes that the metabolism of β-nicotyrine results in the

formation of reactive and potentially toxic intermediates[115;116]. More recently, β-nicotyrine has been reported to be the most potent inhibitor of P450 2A6 activity among a series of nicotine related alkaloids and metabolites with a K_i of 0.37 μM [90]. In support of this, we have shown here that β-nicotyrine has a three-fold higher binding affinity for P450 2A6 than does nicotine. The previous study also suggested that β-nicotyrine was a mechanism based inactivator and time dependent inactivation was reported, however no further characterization of the observed inactivation was provided. Here we have confirmed that β-nicotyrine is an irreversible inactivator of P450 2A6 and also provided evidence that supports the formation of an apoprotein adduct. Alternatively, the observed inactivation could be the result of a tightly bound metabolite not removed by dialysis or the conditions of our size exclusion column.

Both P450 2A6 and P450 2A13 are inactivated during nicotine metabolism [77]. The observed inactivation of both enzymes appears to be mediated by a metabolite of the nicotine 5'-iminium ion, that is, nicotine-mediated inactivation requires at least two sequential oxidations of nicotine. β-nicotyrine is a urinary metabolite of nicotine in some species, and forms from the nicotine 5'-iminium ion in vitro [108;115]. We report in Chapter 3 that β-nicotyrine is formed during P450 2A6-mediated nicotine metabolism. Here, we report that β-nicotyrine is an inactivator of P450 2A6 and hypothesize that inactivation of the enzyme by nicotine may be due to the formation and successive metabolism of β-nicotyrine. Prior to our studies, metabolism of β-nicotyrine by human enzymes, either P450 2A6 or P450 2A13, had not been studied. However, in the rabbit, P450-catalyzed metabolism results in the formation of two pyrrolinones that likely form by way of an

unstable epoxide of the pyrrole ring. This epoxide is one possible candidate for the metabolite that inactivates P450 2A6. Experiments characterizing both P450 2A6 and P450 2A13-catalyzed β-nicotyrine metabolism are presented in Chapter 3 and the products are described therein.

Menthofuran and β-nicotyrine irreversibly inactivated P450 2A6 but not P450 2A13. Consistent with earlier studies of menthofuran inactivated P450 2A6 [105] our data supported the conclusion that modification of the apoprotein, not the heme moiety, is the likely mechanism of inactivation. Like β-nicotyrine, menthofuran is metabolized to a reactive epoxide that may contribute to protein adduct formation [48]. Menthofuran is a more potent inactivator of P450 2A6, with a higher $K_{I(inact)}$ and a lower $t_{½}$, than β-nicotyrine. However, the calculated partition ratio for menthofuran-mediated inactivation was 3.5 times higher than for β-nicotyrine-mediated inactivation. Therefore, despite the relatively high $K_{I(inact)}$ for β-nicotyrine inactivation of P450 2A6, its oxidation more frequently generates a reactive metabolite that inactivates P450 2A6 than does menthofuran.

2.5 Conclusion

In summary, β-nicotyrine but not menthol is likely to impact nicotine metabolism and possibly NNK metabolism in smokers. Menthol was a modest inhibitor of P450 2A6 and P450 2A13. Whereas β-nicotyrine, which is present in tobacco or generated as a metabolite of nicotine, was an effective inhibitor of both P450 2A6 and P450 2A13. More importantly, β-nicotyrine inactivated P450 2A6, the primary catalyst of nicotine metabolism. Interestingly, neither β-nicotyrine nor menthofuran, the potent mechanism based inactivator of P450 2A6, are inactivators of P450 2A13.

Experiments are on-going to characterize the mechanistic basis for this, since it may be useful in the design of P450 2A6 inhibitors and help to elucidate the pathway responsible for the inactivation of P450 2A6 by nicotine.

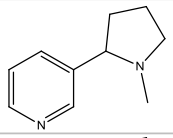
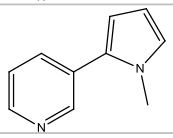
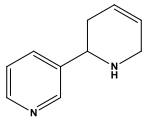
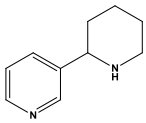
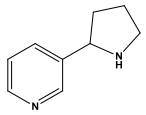
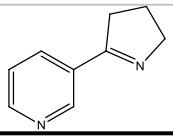
Chapter 3

P450 2A catalyzed β-nicotyrine metabolism and β-nicotyrine as a metabolite of P450 2A6 catalyzed nicotine metabolism.

3.1 Intro

Nicotine is the major pharmacologically active and most abundant alkaloid found in tobacco and thus, has been extensively studied. There are a number of related alkaloids in tobacco that are present at small quantities relative to nicotine, which have been subject to much less study (Table 3-1). One of these alkaloids, β-nicotyrine, is also reported to be generated during pyrolysis [41]. We have recently confirmed that β-nicotyrine is a mechanism-based inactivator ("suicide" inhibitor) of P450 2A6, the primary catalyst of nicotine metabolism [117]. Pharmacologically blocking nicotine metabolism has been reported to reduce the extent of smoking [88;89]. Therefore, if compounds in tobacco block nicotine metabolism, characterizing the exposure of smokers (and other tobacco users) to these compounds is critical to understanding their influence on the continued use of tobacco.

Table 3-1: Alkaloids found in tobacco and their estimated amounts in tobacco smoke.

Compound	Structure	Amount (mg)
Nicotine		0.8-2.85[a,b]
β-nicotyrine		0.004- 0.04[b]
Anabasine		0.002- 0.02[b]
Anatabine		0.002-0.02[a,b]
Nornicotine		0.25[a]
Myosmine		0.013-0.033[b]

[a] Pakhale et. Al., 1998 [42]
[b] Baker et. al., 1999 [41]

The primary pathway of nicotine metabolism is conversion to cotinine. The first step is the P450-catalyzed 5'-oxidation of nicotine (Figure 3-1). The nicotine $\Delta^{5'(1')}$iminium ion (iminium ion) product of this reaction is then further oxidized to cotinine. This oxidation may be carried out by the cytosolic enzyme aldehyde oxidase [34]. However, P450 2A6 and P450 2A13 also catalyze this reaction[72; 68]. In smokers, P450 2A6 in the liver is the primary catalyst of the metabolism of nicotine to cotinine. Cotinine can then be further oxidized to 3'-hydroxycotinine or 5'-hydroxycotinine [118]. In

urine of smokers with typical P450 2A6 activity, the summation of cotinine and subsequent cotinine metabolites account for more than 80% of a consumed nicotine dose[34].

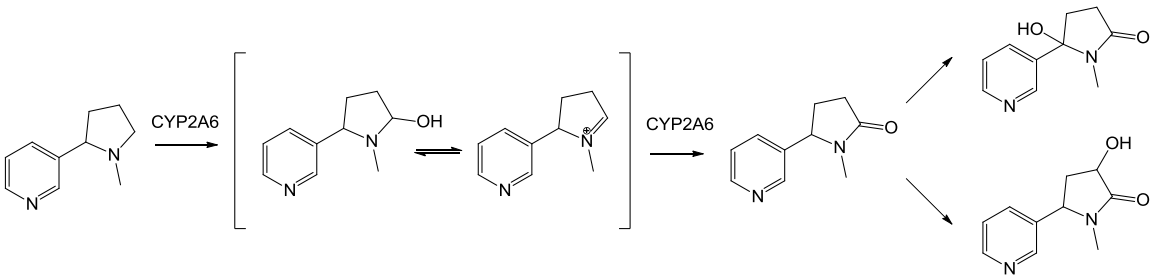


Figure 3-1: Nicotine oxidation to cotinine, 3'-hydroxycotinine and 5'-hydroxycotnine.

Several years ago, it was reported that nicotine inactivated both P450 2A6 and the closely related enzyme, P450 2A13. More specifically, as nicotine was metabolized, enzyme activity was lost [77]. The observed inactivation was dependent on NADPH, time and nicotine concentration [117]. Additionally, inactivation is observed when the iminium ion is metabolized to cotinine. This lead to the suggestion that the species responsible for the observed nicotine inactivation was generated from the nicotine $\Delta^{5'(1')}$iminium ion. Knowing that β-nicotyrine metabolism results in inactivation of P450 2A6, we hypothesized that β-nicotyrine is a nicotine metabolite that plays a role in nicotine-mediated inactivation of P450 2A6. Specifically, we expect that β-nicotyrine is an intermediate found during P450 2A6-mediated nicotine metabolism and that subsequent metabolism of β-nicotyrine would then lead to the inactivation of the enzyme.

Little is known of β-nicotyrine metabolism in smokers, however previous *in vitro* studies have indicated that β-nicotyrine is metabolized rapidly by rabbit, guinea pig,

mouse and rat liver homogenates in a process requiring NADPH and molecular oxygen [119]. Furthermore, unchanged β-nicotyrine could not be detected in the urine of humans following a 2 mg oral dose, suggesting β-nicotyrine is metabolized extensively in humans [119].

The metabolism of β-nicotyrine by rabbit liver and lung has been studied [48;115] and a pathway for its metabolism has been proposed (Figure 3-2). β-nicotyrine was converted to two pyrrolinones (1-methyl-5-(3-pyridyl)-3-pyrrolin-2-one and 1-methyl-5-(3-pyridyl)-4-pyrrolin-2-one) (**4**, **5**), which easily decompose through autooxidation to the hydroxypyrrolinone (5-hydroxy-1-methyl-5-(3-pyridinyl)-3-pyrrolin-2-one) (**6**). The pyrrolinone metabolites are likely formed by way of an epoxide. Chemical and spectral data have implicated 2-hydroxy-β-nicotyrine as a key intermediate (**3**). Under acidic conditions, the pyrrolinones have been shown to undergo hydration to form 5'-hydroxycotinine (**7**). The epoxide may contribute to the β-nicotyrine-mediated inactivation of P450 2A6 that we have reported [117].

The aim of this study was two-fold: to determine if β-nicotyrine is a metabolite of P450 2A6 or P450 2A13-mediated nicotine metabolism and to characterize β-nicotyrine metabolism by P450 2A6 and P450 2A13.

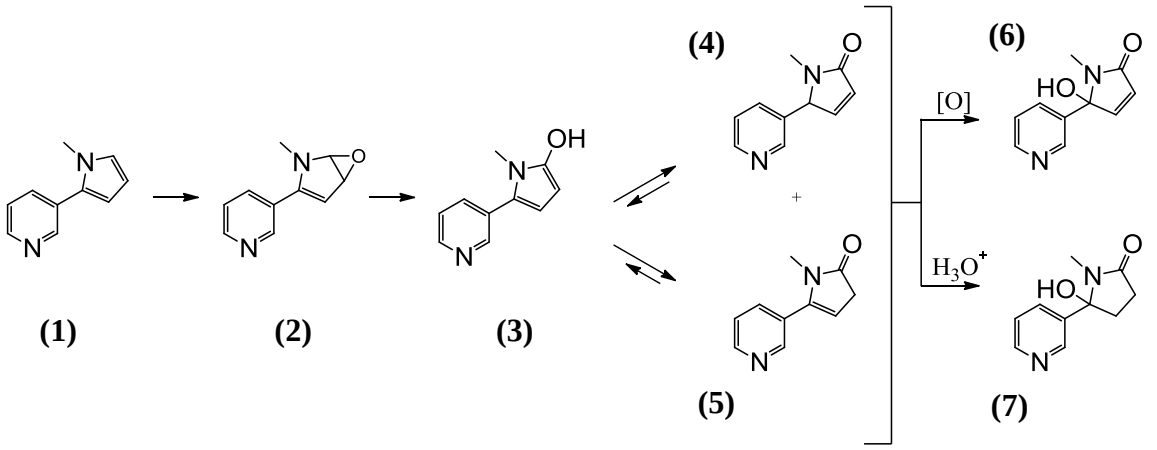


Figure 3-2. Proposed pathway for β-nicotyrine metabolism

3.2 Materials and Methods

3.2.1 Chemicals

[^{3}H]-β-nicotyrine (^{3}H on the pyridine ring, non-specific position) (20 Ci mmol-1, 99% purity) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Nicotine $\Delta^{5'(1')}$ iminium ion was synthesized as described previously (Peterson et al., 1987). 5'-Hydroxycotinine, *cis*- and *trans*-3'-hydroxycotinine, and [CD_3]-cotinine (cotinine-D_3) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

3.2.2 Enzymes

Rat NADPH-P450 oxidoreductase and P450 2A6 and P450 2A13 were expressed in *Escherichia coli* and purified as previously described [68;120]. P450 2A6, P450 2A13 and oxidoreductase were reconstituted in the presence of lipid for 45 min at 4 °C. Then, 50 mM Tris buffer, pH 7.4 and catalase were added to give a final P450 to reductase ratio of 1:1, 0.2 µg lipid/pmol P450, and 60 U/µl catalase.

3.2.3 Metabolism of nicotine and iminium ion

Nicotine or iminium ion (5 or 200µM) was incubated with reconstituted P450 2A6 or P450 2A13 (0.25 or 1 µM) in a final volume of 50µl. Reactions were initiated by the addition of a NADPH-generating system (0.4 mM NADP+, 100mM glucose-6-phosphate, and 0.4 units ml-1 glucose-6-dehydrogenase), were incubated for 0, 1 or 12 minutes at 37°C then terminated by the addition of 5 µl 15% trichloroacetic acid.

3.2.4 Metabolism of ^{3}H-β-nicotyrine

Total β-nicotyrine metabolism was determined using ^{3}H-β-nicotyrine and radioflow HPLC analysis. The method used was a modification of a previously described HPLC system (Chapter 2). For product determination, reconstituted P450 2A6 or P450 2A13 (1 µM) were incubated with 20 µM ^{3}H-β-nicotyrine (50 µCi mmol^{-1}), 100 mM Tris buffer (pH 7.4), NADPH-generating system (0.4 mM NADP+, 100 mM glucose-6-phosphate, and 0.4 units ml^{-1} glucose-6-dehydrogenase) in a final volume of 200 µl for 5

minutes at 37°C. Similar conditions were used to determine kinetic parameters however less P450 (0.2 µM) and varied amounts of ^{3}H-β-nicotyrine (0.25, 0.5, 0.75, 1, 2, 5, 10 or 20 µM) were allowed to incubate for shorter times (30 sec). The short reaction time for kinetics experiments was chosen to minimize the occurrence of inactivation. Reactions were terminated by addition of 20 µl 15% trichloroacetic acid. All analyses were conducted in triplicate. Products were analyzed by radioflow HPLC with a Phenomonex Gemini C18 (5µm, 250 x 4.60 mm) column. A flow rate of 1 ml min^{-1} was used. Compounds were separated using a linear gradient of aqueous 20 mM ammonium bicarbonate (pH 7.4) (A) and acetonitrile (B). The gradient moved from 96% A: 4% B to 94% A: 6% B in 20 min, then 80% A: 20% B in 10 min, 65% A: 35% B in 5 min, 60% A: 40% B in 5 min and held at 40% B for 5 additional min. Radioactive compounds were detected using a β-RAM radioflow detector (IN/US Systems, Tampa, FL) with a scintillant flow rate of 2.4 ml min^{-1} [Monoflow 5, National Diagnostics, Atlanta, GA]. To assess product stability, a single sample was analyzed multiple times over a period of 20 hours. During the time between analyses, samples were kept in a closed tube at room temperature.

Radioactive peaks were collected and concentrated under nitrogen. Samples were re-injected to assess purity. A portion of each of the collected metabolites were placed in acidic conditions (pH 1) by addition of 30 µL of concentrated hydrochloric acid and allowed to incubate for 3 hours at room temperature. The collected, ^{3}H-labeled products were co-injected with β-nicotyrine, 5'-hydroxy cotinine, 3'-hydroxy cotinine standards, detected by absorbance at 260 and 284 nm.

After determination of [^{3}H]-analyte retention times by radioflow HPLC, a sample generated using identical conditions but non-radioactive β-nicotyrine was injected and metabolites were collected. Each collected fraction was concentrated under nitrogen and major products in collected fractions were determined by LC-MS (as described below). Subsequent product ions were identified by LC-MS/MS of the precursor ion (as described below).

3.2.5 LC-MS and LC-MS/MS analysis of β-nicotyrine metabolites

LC-MS/MS analysis was performed on an Agilent 1100 series capillary HPLC system (Agilent Technologies) interfaced to a Thermo Fisher Scientific Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific). The samples (2–8 μl) were injected onto a Zorbax SB-C18 0.5 mm × 150 mm, 5 μm particle size (Agilent Technologies).

Compounds were eluted with a gradient that held at 99.9% water (0.1% formic acid) for five minutes, followed by a linear gradient from 99.9% water (0.1% formic acid) to 69.9% water: 30% acetonitrile: 0.1% formic acid in 20 minutes at a flow rate of 15 μl/min. The column was operated at 40 °C. For the first 2.5 minutes, the eluant was diverted to waste before directing the flow into the source of the mass spectrometer.

LC-MS and LC-MS/MS were performed on the same system under similar conditions. The ESI source was operated in the positive ion mode with a collision energy of 25 or 30 V, the s-lens RF amplitude of 75, the collision gas pressure set to 1.0 mTorr

and the source CID collision energy set to 25-30V. For LC-MS/MS, selected reaction monitoring (SRM) of the mass transitions for the various analytes were used for identification. The scan width was 0.30 (*m/z*) and the scan time 0.05 s and the peak widths for Q1 was 0.50 and Q3 was 0.70. The mass transitions were as follows: nicotine (*m/z* 163 → 130 and *m/z* 163 → 117), cotinine (*m/z* 177 → 98 and *m/z* 177 → 80), β-nicotyrine (*m/z* 159 → 117 and *m/z* 159 → 144), Isomeric pyrrolinones of β-nicotyrine (*m/z* 175 → 117 and *m/z* 175 → 132), β-nicotyrine hydroxypyrrolinone(*m/z* 191 → 80 and *m/z* 191 → 132), and 5'-hydroxycotinine (*m/z* 193→80, *m/z* 193→134 and *m/z* 193→149)

3.2.6 Quantification of cotinine and β-nicotyrine

A standard curve was generated from independent solutions of increasing concentrations of cotinine (0.125-5 µM) that contained 1 µM cotinine-D_3. The same amount of cotinine-D_3 was added to each sample. Eight microliters of each standard or sample were injected on the instrument. Cotinine was quantified by comparing the peak area ratios of cotinine/cotinine-D_3 to those generated from the standard curve. β-Nicotyrine internal standard is not available. Therefore, a standard curve of β-nicotyrine (1-50 nM) was prepared with cotinine-D_3 (1 µM) and β-nicotyrine was quantified in a similar manner to cotinine (Figure 3-2). This method was validated as cotinine and β-nicotyrine responses were linear within the range of concentrations used in the standard curves.

3.2.7 Data Analysis

K_m and V_{max} values were determined using the Sigma Plot kinetics program from Systat Software Inc. (Chicago, IL). Statistical differences between mean K_m or V_{max} values were determined using a Chi square test.

3.3 Results

3.3.1 Nicotine and Iminium Ion Metabolism

To determine if β–nicotyrine is a metabolite of nicotine or iminium ion, complete reaction mixtures generated from each of these compounds by either P450 2A6 and P450 2A13 were analyzed by LC-MS/MS. By monitoring mass transitions for β–nicotyrine, we have confirmed that β–nicotyrine is generated during the P450 2A6-mediated metabolism of nicotine and iminium ion in an NADPH-dependent manner (Figure 3-3, A and B, respectively). Retention time and relative abundance of the two mass transitions were identical to β–nicotyrine standard (Figure 3-3, C). The amount of standard in Figure 3-1, C is low (1 fmol) to illustrate the limit of quantification. In contrast, no β–nicotyrine was detected during the P450 2A13-mediated metabolism of either nicotine or iminium ion (Figure 3-3, D and E respectively) suggesting that none was formed or it was present at lower amounts than could be detected in low-level standards with the level of background present in control samples (Figure 3-3, F).

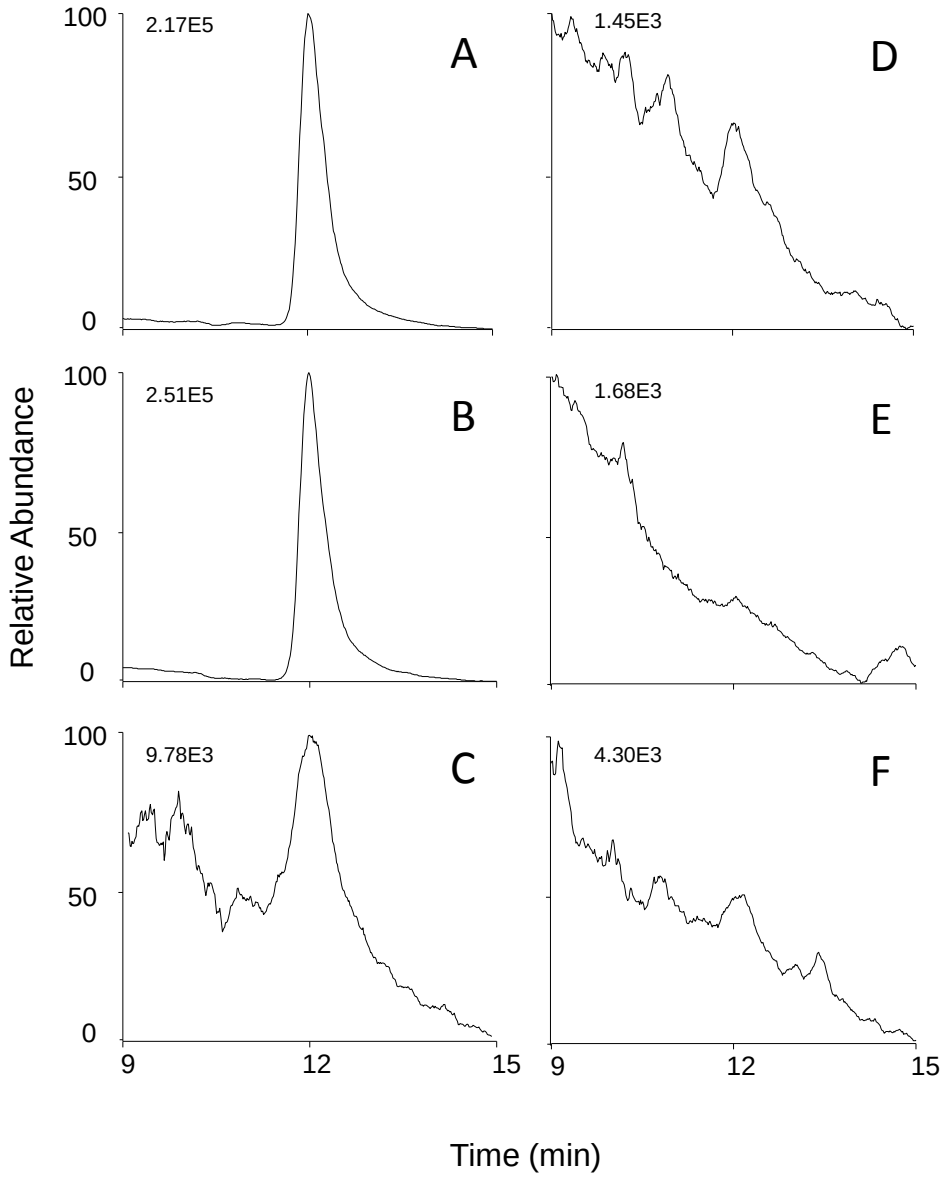


Fig 3-3. LC/MS-MS analysis of nicotine metabolites generated by P450 2A6. LC-MS/MS analysis for *m/z* showing *m/z* 159→ 117 and 159→144 mass transitions from P450 2A6-mediated nicotine (A) and iminium ion (B) metabolism, the 1 fmol β–nicotyrine standard (C), P450 2A13-mediated nicotine (D) and iminium ion (E) metabolism and a P450 2A6 control sample that contained no NADPH (F).

Using a standard curve normalized to cotinine-D_3 used as an internal standard in the reactions, the amount of β–nicotyrine generated during P450 2A6-mediated nicotine

and iminium ion metabolism was quantified (Figure 3-4). Formation of β–nicotyrine was dependent on time, NADPH and P450 2A6 concentration (Table 3-2 A). Under the conditions used, the amount of β–nicotyrine generated was between 2% and 12% of the cotinine generated in the same experiment (Table 3-2 A and B).

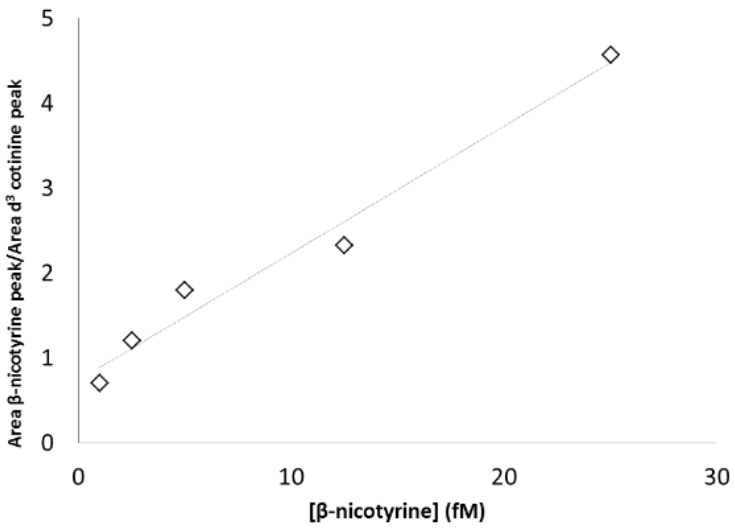


Fig. 3-4 Standard curve used to quantify β-nicotyrine. 1 µl of β-nicotyrine standards (1, 3.125, 6.25, 12.5, and 25 nM) containing 10µM d^3-cotinine were used to generate standard curve.

Table 3-2. Amounts of β-nicotyrine and cotinine generated during P450 2A6-mediated nicotine or iminium ion metabolism.

	β–nicotyrine/P450 2A6 (fmol/pmol)	
	Time (min)	
Substrate	1	12
Nicotine (200 µM)	10.4	183.6
Nicotine (5 µM)	ND	12.0
Iminium Ion (200 µM)	17.4	141.8
Iminium Ion (5 µM)	ND	47.2

	Cotinine/P450 2A6 (fmol/pmol)	
	Time (min)	
Substrate	1	12
Nicotine (200 µM)	78.9	767.2
Nicotine (20 µM)	ND	137.0
Iminium Ion (200 µM)	537.6	1946.5
Iminium Ion (20 µM)	231.4	839.4

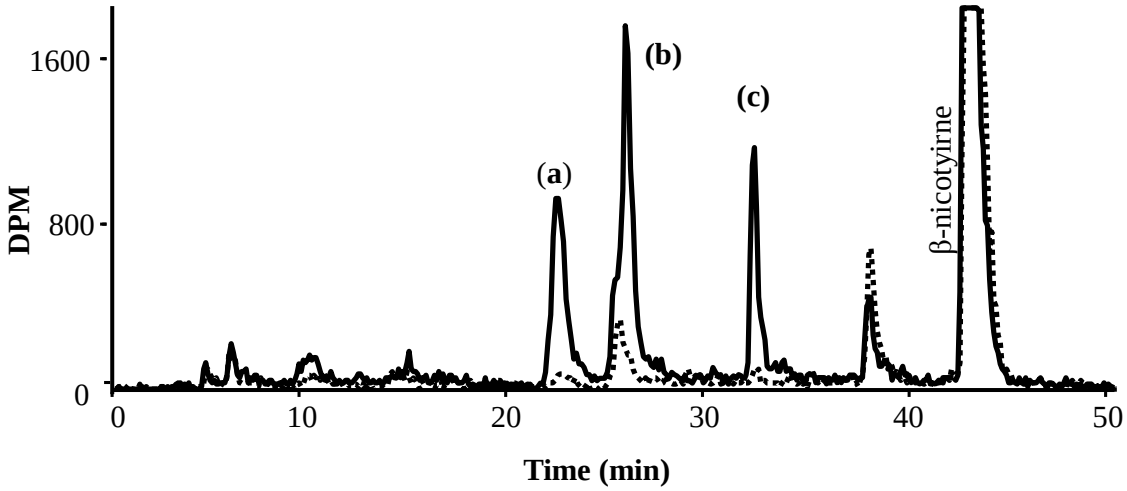


Figure 3-5. Radioflow HPLC analysis of P450 2A6 catalyzed metabolism of β-nicotyrine. P450 2A6 was incubated with β-nicotyrine in the presence (solid line) or absence (dashed line) of NADPH.

3.3.2 P450 2A6 and P450 2A13-mediated β-metabolism

The relative metabolism of ^{3}H-β-nicotyrine by P450 2A6 and P450 2A13 was analyzed by radioflow HPLC. P450 2A6-mediated metabolism of β-nicotyrine resulted in four radioactive peaks. The peak with an elution time of 38 minutes appeared in the control sample and was time-dependent but not NADPH-dependent. The peak with a retention time of 26 minutes eluted very close to a peak that also appeared in the control sample. Five-second fraction collection of the time spanning this peak and subsequent LC-MS/MS analysis of these fractions demonstrated that the compound in the control sample that appears at 26 min was also present in the metabolized sample but was distinct from product (b). Therefore, the shoulder seen on the left side of peak (b) was not a metabolite and was excluded from quantification (Figure 3-5). Thus, three product peaks

(retention times were 23 min (**a**), 26 min (**b**) and 32 min (**c**)) were considered for further analysis. Metabolite **a** co-eluted with the 5'-hydroxycotinine standard (data not shown). P450 2A13-mediated metabolism of β-nicotyrine generated the same metabolites.

It was observed that the relative amounts of the metabolites formed during identical experiments carried out on different days resulted in inconsistent product ratios. It became obvious that the product ratio variation was dependent upon the time between reaction termination and sample analysis. Therefore, to assess product stability, a sample was allowed to sit at room temperature in capped tubes before analysis. After five hours, the amount of metabolite **c** decreased and the amount of **a** and **b** increased (Figure 3-6). This indicates that non-enzymatic conversion of metabolite **c** to **a** and **b** is occurring.

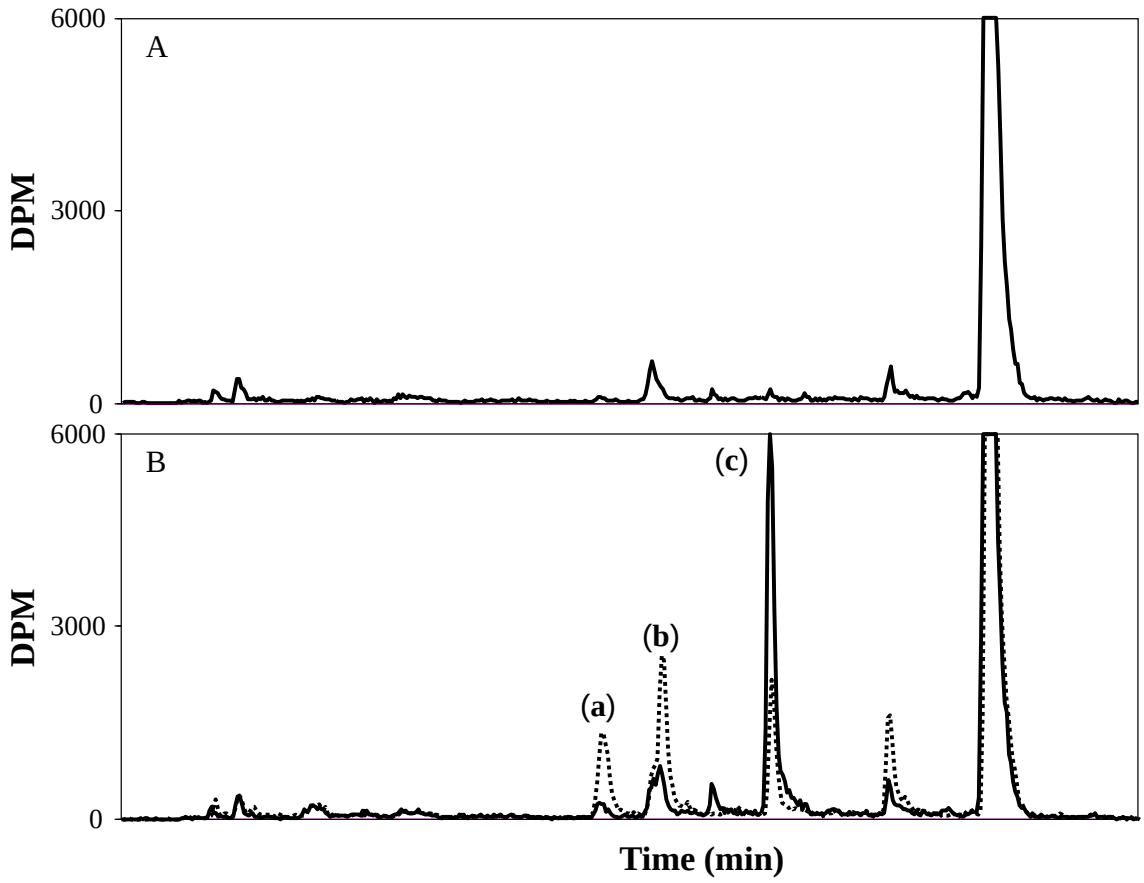


Fig 3-6. Radioflow HPLC analysis of β-nicotyrine metabolites. . A. Control Sample (-NADPH) is shown in panal (A) and sample containing metabolites (a), (b) and (c) either 0 hours (solid line) and 5 hours (dashed line) at room temperature.

After determination of [^{3}H]-analyte retention times by Radioflow HPLC, a sample was injected a second time and fractions were collected for the [^{3}H]-metabolites **(a)**, **(b)** and **(c)** elution times. A similar but non-radioactive sample was also injected and, based on retention time, metabolites **(a)**, **(b)** and **(c)** were collected. These collected samples were used to further characterize the three product peaks. Additionally, a portion of the non-radioactive reaction was analyzed directly by LC-MS. This analysis identified four

metabolite peaks. Mass to charge ratios for each of these were *m/z* 159, *m/z* 175, *m/z* 191 and *m/z* 193.

Several pieces of evidence indicate that metabolite **a** is 5'-hydroxycotinine. First, as mentioned above, **a** co-eluted with 5'-hydroxycotinine standard during LC/Radioflow/UV analysis. Secondly, collected metabolite peak **a** had a *m/z* of 193 (Figure 3-9, A) which is consistent the molecular weight of 5'-hydroxycotinine. Finally, when an LC-MS/MS trace of **a** is compared to that of the 5'-hydroxycotinine standard, retention time and relative abundance of mass transitions are identical (Figure 3-7). 5′-Hydroxycotinine is in equilibrium with 4-(3-pyridyl)-4-oxo-N-methylbutyramide (keto amide) (Figure 3-8) that eluted as separate peaks (retention times 3.7 and 5.2 min). Both peaks are detected in collected metabolite **a** and the standard. The keto amide has two major fragments (*m/z* 193→80, *m/z* 193→134). Major fragments of 5'-hydroxycotinine include the two that are observed for the keto amide as well as an additional fragment at *m/z* 193→149 (Figure 3-9, A, inset).

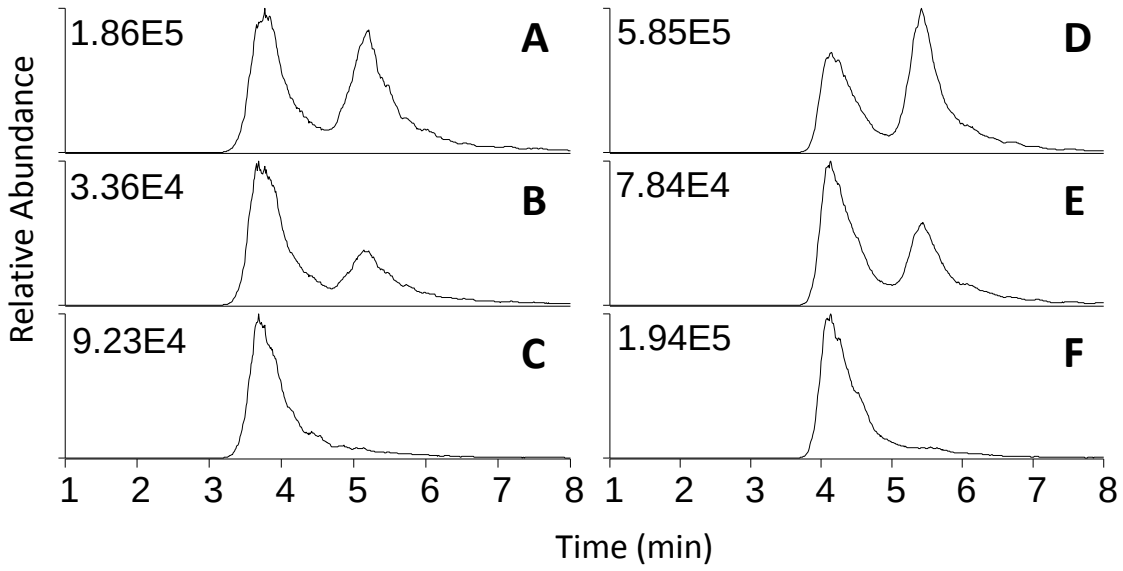


Figure 3-7. **LC-MS/MS of** 5'**-hydroxycotinine and collected metabolite a**. LC-MS/MS using SRM of 5'-hydroxycotinine standard (A-C) and collected metabolite **a** (D-F). Mass transitions monitored are *m/z* 193→80 (A,D), *m/z* 193→134 (B,E) and *m/z* 193→149 (C,F).

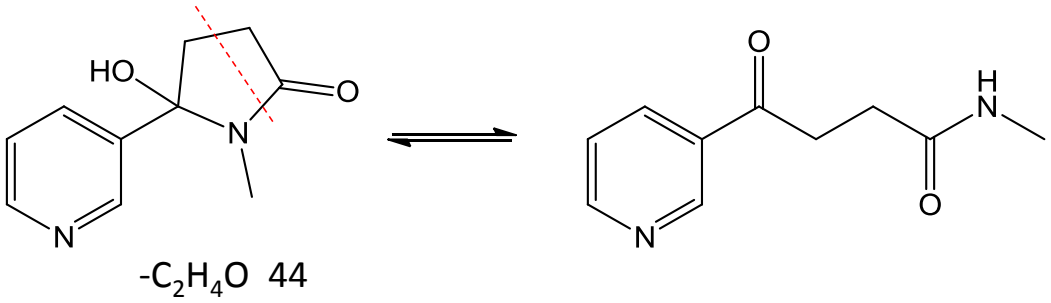


Figure 3-8. 5'-hydroxycotinine in equilibrium with the keto amide. Dotted line indicates bond breaking resulting in *m/z* 149.

The parent ion for metabolite **b** was *m/z* 191 (Figure 3-9, B). This is the mass of the hydroxypyrrolinone (**6**) (Figure 3-2) consistent with the identification of it as a metabolite of β-nicotyrine that underwent two oxidations. Product ion spectra from *m/z*

191 $[M - H]^+$ contained the protonated pyridine fragment, *m/z* 80. Because the pyridine ring is present in all of the proposed metabolites of β-nicotyrine, this fragment is not diagnostic (Figure 3-9, B inset).

The mass of metabolite **c** had a precursor *m/z* of 175, corresponds to the mass of a β-nicotyrine metabolite that underwent one oxidation (Figure 3-9, C). One oxidation of β-nicotyrine generates the 2-hydroxy-β-nicotyrine (**3**) or the isomeric pyrrolinones (Figure 3-2, **4** and **5**). The product ion spectra from *m/z* 175 $[M - H]^+$ contained four major fragments (Figure 3-5, inset). Two of these fragments support the identification of **c** as the pyrrolinones; the *m/z* 118 corresponding to the loss of C_2H_2NO and the *m/z* 147 corresponding to the loss of CO are generated from either of the pyrrolinones (Figure 3-9 C).

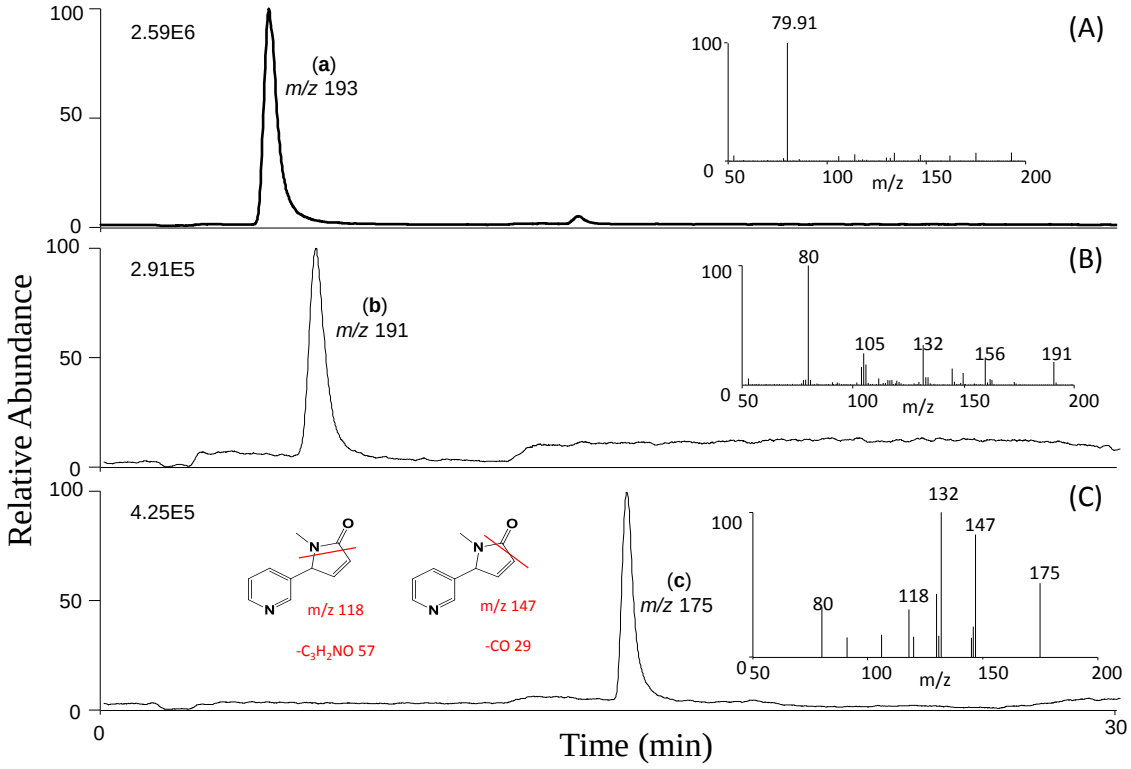


Figure 3-9. LC-MS/MS analysis of collected β-nicotyrine metabolites a, b and c. Total ion chromatographs are shown with parent masses labeled next to the major product peak. MS/MS spectra are shown as insets.

A fraction of [^{3}H]-metabolite **c** was incubated at room temperature under acidic conditions. When re-injected on the HPLC system, the analyte peak co-eluted with the 5'-hydroxycotinine standard (Figure 3-10). Based on previous work by Liu et al, 1999 and the relevant chemistry, this conversion of metabolite **c** to 5'-hydroxycotinine further supports the identity of **c** as a pyrrolinone [48].

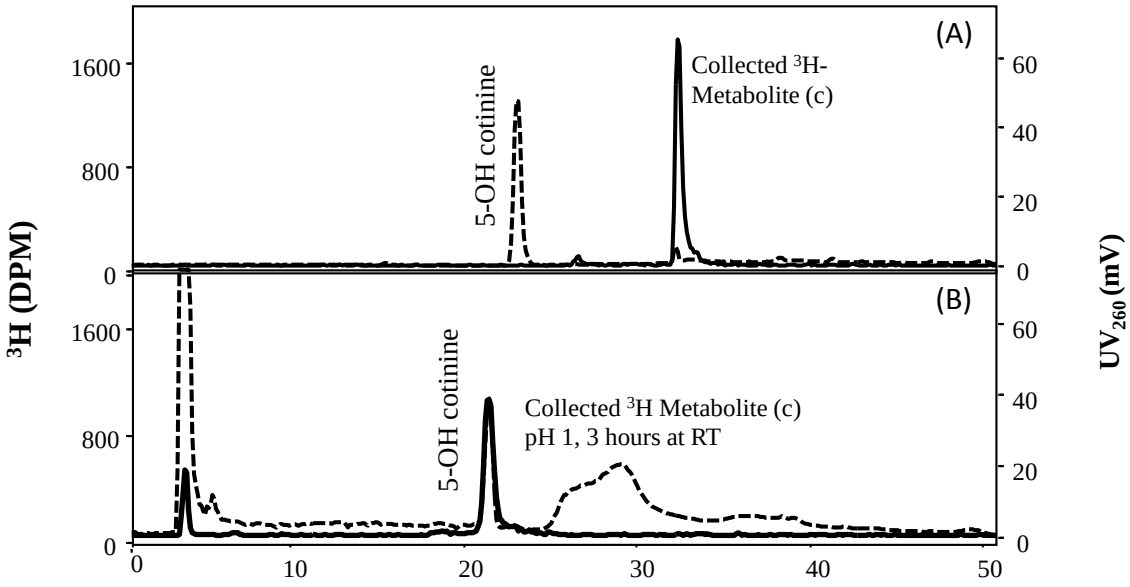


Fig 3-10. Radioflow HPLC analysis of metabolite (c) with and without acid. Collected [^{3}H]-metabolite (c) was either analyzed by HPLC/Radioflow (Top panel, solid line) or incubated in acidic conditions analyzed by HPLC/Radioflow (bottom panel, solid line). Samples in both cases were co-injected with 5'-hydroxycotinine and monitored by UV (260 nm) (dashed lines).

Due to the conversion of metabolite **c** to metabolite **a** and the variability in quantification of metabolite **b** due to its autooxidation from metabolite **c**, all three products were summed to determine the rate of the initial β-nicotyrine oxidation. Kinetic

constants were determined for both P450 2A6 and P450 2A13-mediated metabolism of β-nicotyrine (Figure 3-11). Both P450 2A6 and P450 2A13 metabolized β-nicotyrine very quickly as indicated by the relatively high V_{max} values (21 and 37 pmol product/min/pmol, respectively). The calculated K_m values for P450 2A6 and P450 2A13-mediated β-nicotyrine metabolism (4.4μM and 5.0μM, respectively) are similar to the reported K_m for coumarin 7-hydroxylation (6.0 μM and 3.7 μM, respectively [75;111].

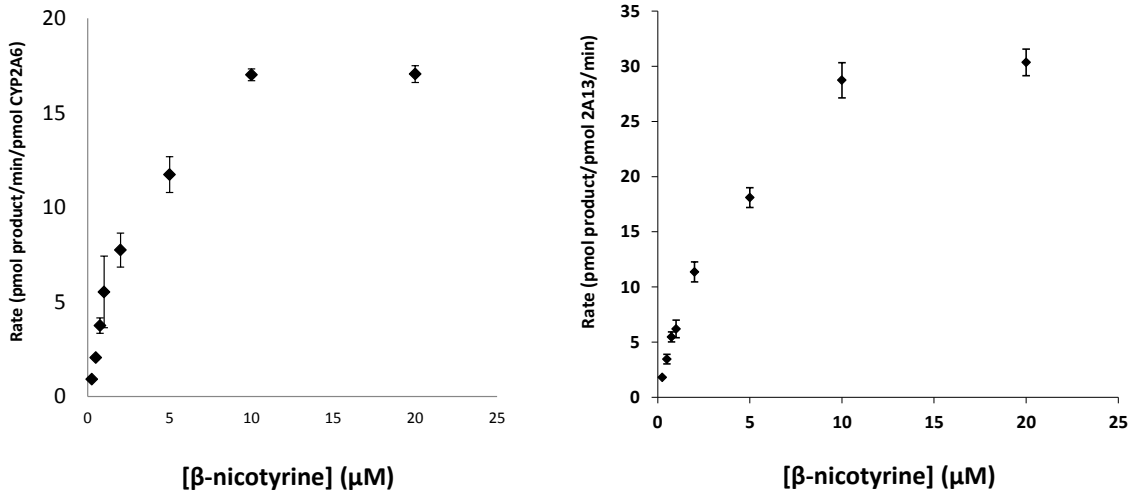


Fig 3-11. P450 2A6 and P450 2A13-mediated metabolism of β-nicotyrine. Kinetic curves for P450 2A6-mediated (Left) and P450 2A13-mediated (Right) metabolism of β-nicotyrine.

3.4 Discussion

Assessing a smoker's exposure to β-nicotyrine and understanding its subsequent influences on smoking behavior are necessary to gauge if this compound ought to be monitored in tobacco products. Additionally, knowing the mechanism of the observed β-nicotyrine-mediated P450 2A6 inhibition could aid in the design of a drug for smoking

cessation. This study set out to determine if exposure could occur due to the generation of β-nicotyrine from nicotine and characterize the P450 2A-mediated metabolism of β-nicotyrine.

β-Nicotyrine is metabolized to the same products by both P450 2A6 and P450 2A13. Three products were identified; two of them form as a result of decomposition of the first. Evidence supporting the primary metabolite as a mixture of two isomeric pyrrolanones (**4** and **5**) is presented here (Figure 3-2). This finding is supported by previous work that identified these compounds as metabolites of β-nicotyrine in rabbit liver and lung microsomes [48;115]. One of the two decomposition products is likely 5-hydroxyl-1-methyl-5-(3-pyridinyl)-3-pyrrolin-2-one (**6)** and the other is 5'-hydroxycotinine (**7**). While we have shown here that 5'-hydroxycotinine can form by autooxidation of the pyrrolanones (**4** and **5**), our work does not exclude the possibility that it is also formed by P450-mediated oxidation. This distinction matters with regard to the accuracy of the calculated kinetic parameters of the initial β-nicotyrine oxidation. In an ideal world, synthesized pyrrolinone could be incubated with P450 2A6 or P450 2A13 to determine if it is a substrate for these enzymes, however there are two problems with this experiment. The first is a practical issue. While it is possible to synthesize a mixture of the pyrrolinones, they are chemically unstable and therefore would be difficult to quantify and use before decomposition occurred [48]. One must consider the mechanism of substrate turnover to understand the second issue. Catalysis of sequential oxidations, where the substrate does not leave the active site, is not unusual in cytochrome P450 reactions. Sequential oxidation is seen with P450 2A6-mediated nicotine and

diethylnitrosamine metabolism[121-123]. If sequential oxidation occurs during P450 2A6-mediated β-nicotyrine metabolism, the rate of pyrrolinone metabolism would not include binding of the substrate to the enzyme. Kinetic parameters generated from an experiment that started with the pyrrolinone would include this, possibly limiting, binding step.

β-Nicotyrine is generated during P450 2A6-mediated metabolism of nicotine and iminium ion. Here we have estimated that the amount of β-nicotyrine generated is between 3% and 24% of the cotinine generated. While this number is to be considered an estimate, as it does not account for subsequent metabolism of β-nicotyrine or cotinine, a rough extrapolation can be made to an *in vivo* situation. Assume a smoker took in 1 mg (5.6 μmol) of nicotine per cigarette and 80% of that was converted to cotinine. This leaves 4.5 μmol of cotinine. If the amount of β-nicotyrine generated is three percent of the cotinine, 136 nmol of β-nicotyrine would be generated per cigarette. According to the partition ratio calculated for β-nicotyrine inactivation of P450 2A6 [117], ten percent of that would lead to inactivation. Loss of 13 nmol of P450 2A6 activity could have a significant impact on nicotine metabolism and subsequent smoking behavior. Albeit, this assumes optimal conditions and a linear relationship between *in vitro* to *in vivo* work, it has been included to highlight that further study of β-nicotyrine impact on nicotine metabolism is warranted.

It is curious that β-nicotyrine was not detected during P450 2A13-mediated metabolism of nicotine or iminium ion. The simple explanation for this would be that no β-nicotyrine is generated during P450 2A13-catalyzed nicotine metabolism. While there is a high degree of similarity between these two enzymes, there are examples where they

generate different products or product ratios from the same substrate. One well-characterized example of this is P450 2A6 and 2A13-mediated coumarin metabolism. P450 2A6 solely generates 7-hydroxycoumarin whereas P450 2A13 produces 7-hydroxycoumarin and the coumarin 3,4-epoxide [75]. Another example of a difference between these two enzymes is cotinine metabolism. While norcotinine is the major product of P450 2A6-mediated cotinine metabolism, it is a very minor product of P450 2A13-catalyzed cotinine metabolism [124]. It stands to reason that, if β-nicotyrine is a much more minor product of P450 2A13-mediated than P450 2A6-mediated nicotine metabolism, and we know that β-nicotyrine is rapidly metabolized by P450 2A13, any β-nicotyrine generated would be so rapidly turned over it would likely be missed under the conditions of our analysis. A follow up experiment looking for the β-nicotyrine pyrrolinones, hydroxy-pyrrolinones and 5'-hydroxycotinine during P450 2A13-mediated nicotine metabolism would distinguish between these two explanations.

3.5 Conclusion

While the metabolic differences between P450 2A6 and P450 2A13 are interesting with regard to our understanding of structure-function relationships in P450 active sites, the big picture relevance of this study is the impact β-nicotyrine may have on P450 2A6-mediated nicotine metabolism. P450 2A6 is primarily responsible for nicotine metabolism *in vivo*. The identification of β-nicotyrine as a product of this metabolism combined with the knowledge that it inactivates P450 2A6 is novel, could potentially impact nicotine metabolism and thus, could be useful in nicotine cessation products.

Chapter 4

Protein modification during inactivation

4.1 Introduction

P450 inhibitors can be categorized as reversible (competitive or noncompetitive), quasi-irreversible, or irreversible in nature. Reversible inhibition usually involves two or more compounds competing for binding at the active site of a P450, with one compound inhibiting the metabolism of the other. In contrast, irreversible P450 inhibitors are catalytically converted to electrophilic reactive metabolites that may interact with the P450 quasi-irreversibly or irreversibly leading to enzyme destruction [104]. The inactivated P450 is catalytically incompetent and must be replenished by newly synthesized protein. The phenomenon is referred to as time-dependent inhibition, mechanism-based inactivation (MBI), and/or suicide inactivation. It should be noted that not every turnover of the inhibitor in question leads to inactivation of the enzyme; generation and release of product can still occur a percentage of the time (Figure 4-1). The accepted model is that inactivation occurs due to a covalent bond formed between the reactive species and the enzyme, however, a very tight-binding intermediate could also fit the criteria for MBI [104]. If the mechanism of inactivation is through a covalent modification, the modification could be on either the heme or the apoprotein.

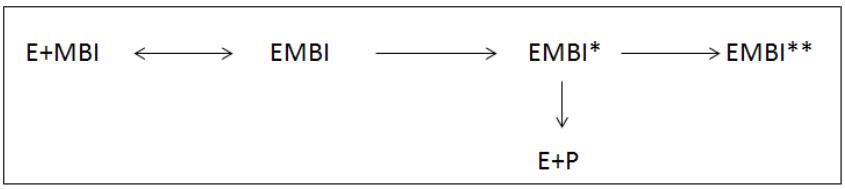


Figure 4-1. Representative pathway to mechanism based inactivation. Enzyme (E) binds to Mechanism Based Inactivator (MBI), converts MBI to reactive intermediate (MBI**) that can subsequently be converted to product (P) or irreversibly bind to the enzyme (EMBI**).

MBI has been useful in the design of potential drugs. Most enzyme inhibitor drugs are reversible inhibitors and so the basis for their effectiveness is the tightness of their binding to the P450. The effectiveness of the drug continues as long as enzyme is complexed with the drug. Because the enzyme concentration is low and fixed, the equilibrium between complexed and non-complexed drug will depend on the concentration of drug and the competing substrate. Concentration of drug will diminish due to metabolism and excretion; concurrent with this, the concentration of enzyme in complex with substrate will increase. Therefore, to maintain effective P450 inhibition, the drug must be re-administered. A drug that acts as a mechanism-based inactivator, however, could form a covalent bond to the enzyme, thereby preventing the dissociation of the inactivator from the enzyme, allowing for less-frequent and lower dosing of the drug. This is particularly appealing as most adverse drug reactions correlate with high therapeutic doses[125]. Unwanted inactivation, however, needs to be considered carefully. Compared to reversible P450 inhibition, drug-induced MBI of P450s presents a greater safety concern because of the increased propensity for drug-drug interactions upon

multiple dosing and the sustained duration of these interactions after discontinued administration of the mechanism-based inactivator[126].

Pharmacologically inhibiting the activity of P450 2A6, the P450 responsible for nicotine metabolism, has been shown to decrease the rate of nicotine elimination and subsequently can reduce the extent of smoking [88;89] The identification of a mechanism-based inactivator specific to this enzyme could provide a very strong drug candidate for smoking cessation. We have previously reported that nicotine, nicotine$^{\Delta 1',5'}$iminium ion or β-nicotyrine metabolism results in inactivation of P450 2A6 [77;117]. We have shown that β-nicotyrine is generated during nicotine metabolism (Chapter 3 of this thesis) leading to the hypothesis that inactivation of P450 2A6 during nicotine and β–nicotyrine metabolism is related. β–Nicotyrine metabolism by P450 2A6 has been studied; two isomeric pyrrolinones and hydroxyl-pyrrolinone of β–nicotyrine have been identified as products (Chapter 3 of this thesis).

As inactivation controls, two known MBIs of P450 2A6 are used in this study; menthofuran, which has been identified as a potent mechanism based inactivator of P450 2A6 and 8-Methoxypsoralen (8-MOP), a known inactivator of P450 2A13 and P450 2A6[106;107]. Menthofuran-mediated loss of P450 2A6 activity was dependent on catalytic turnover and immunohistochemistry analysis provided evidence that the apo-protein was modified [105]. In addition to demonstrating that 8-MOP-mediated inactivation of P450 2A13 was dependent on catalytic turnover and time, mass-spectral evidence of a covalent adduct to the protein was presented. Furthermore, in humans, ingestion of 8-MOP directly reduces smoking in a laboratory setting [88].

In this study, we aim to begin elucidating the chemistry involved in β-nicotyrine-mediated inactivation of P450 2A6. Identifying the mass of a covalent modification and the site of adduction is related to the catalytic mechanism of the enzyme and can help in this aim.

4.2 Materials and Methods

4.2.1 Chemicals and Enzymes

Optima® grade acetonitrile was purchased from Fisher Chemical (Fair Lawn, NJ). Sequencing grade modified trypsin was purchased from Promega (Fitchburg, WI). β-nicotyrine [^{3}H(G)]-β-nicotyrine (20 Ci mmol-1, 99% purity) was purchased from American Radiolabeled Chemicals (St. Louis, MO). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).The enzymes used in this study were heterologously expressed in *Escherichia coli* and purified as described in Chapter 2 of this thesis.

4.2.2 Inactivation

P450 2A6 (0.1 nmol) was reconstituted with reductase (0.1nmol) and lipid (5µg) for 45 min at 4° C (100 µl total volume). The primary reaction mixture contained 1 pmol/ml P450, 1 pmol/ml reductase, 75 ng/ml lipid, 26 U/ml catalase, 20 µM menthofuran or 50 µM β-nicotyrine or 20 µM 8-MOP and 1 mM NADPH (water in control samples) in 50 mM Tris buffer (pH 7.4) (50 µl total volume). The samples were incubated for 5 min at 30 prior to the addition of NADPH. At 5 min following NADPH

addition the control (+NADPH, -Inhibitor), exposed (-NADPH, +Inhibitor) and inactivated (+NADPH, +Inhibitor) samples (10 pmol P450) were injected on reverse phase HPLC and analyzed on the ion trap mass spectrometer.

Inactivation by ^{3}H-β-nicotyrine was carried out under the same conditions except less lipid (10 μg) and β-nicotyrine (20 μM, 1 Ci mmol^{-1}) was used. After ten minutes incubation, samples were injected on a radioflow HPLC with a JupiterProteo4u (250 x 4.60 mm) column. A flow rate of 1 ml min^{-1} was used. Compounds were separated using a linear gradient of 0.1% trifluoroacetic acid (A) and acetonitrile (B). The linear gradient moved from 70% A: 30% B to 30% A: 70% B in 35 min. Radioactivity was detected using a β-RAM radioflow detector (IN/US Systems, Tampa, FL) with a scintillant flow rate of 2.4 ml min^{-1} [Monoflow 5, National Diagnostics, Atlanta, GA].

4.2.3 Effect of trapping agents

Effect of trapping agents on inactivation were determined by co-incubating menthofuran (5 μM) or β-nicotyrine (20 μM) in the primary reaction mixture (as described in above section) with 10 mM glutathione or semicarbazide. The primary reaction mixtures were incubated for 10 min before aliquots were added to the secondary reaction mixture (as in above section).

4.2.4 Tryptic Digestion

Inactivated P450 2A6 (as described in 4.2.2) was diluted 1:4 in nanopure water

and acetonitrile was added to a final concentration of 10% (v/v) MeCN. The pH of the solution was adjusted to 7.5-9.0 by adding 50 mM ammonium bicarbonate containing 1 mM $CaCl_2$. The lyophilized trypsin was solubilized in 50 mM acetic acid. Trypsin was added to the protein solution to a final concentration of ~1:60 protease:protein (w/w) and the digestion was allowed to proceed overnight at 37 °C. The samples were cooled to RT and the pH was adjusted to 3 with glacial acetic acid (1-5 µL). The digested samples were stored at -80 °C until analysis by LC-ESI+-MS/MS.

4.2.5 LC-ESI+-MS analysis of whole protein

Helium was the nebulizing and drying gas (15 psi, 5 L/min) which had a temperature set at 200 °C. Reverse phase LC was performed on an Agilent 1100 capillary HPLC with a Zorbax 300SB-C8 (3.5 µm, 150 x 0.3 mm) (Agilent Technologies, Santa Clara, CA). Products were eluted at a constant flow rate of 12µL/min with the following gradient: hold at 70% A: 30% B for five minutes; linear gradient to 35% A: 64% B in ten minutes; linear gradient to 20% A: 80% B in 15 minutes; linear gradient to 10% A: 90% B in five minutes. Solvent A was 0.1% trifluoroacetic acid in water (v/v) and solvent B was acetonitrile containing 0.1% trifluoroacetic acid. ESI-LC-MS for β-nicotyrine and 8-MOP-inactivated samples were carried out with an Agilent 1100 series LC-MSD Trap SL mass spectrometer operating in positive ion mode. MS scans were acquired with the sheath gas set to 90 (arbitrary units) and the auxillary gas set to 30 (arbitrary units). The spray voltage was 3.5 kV, the capillary voltage 45 V, and the capillary temperature was set at 200. The protein spectra were deconvoluted using the

Data Analysis for LC-MSD Trap Version 3.2 (Bruker, Billerica, MA). The mass spectrometer is located in the Analytical Biochemistry core facility of the Masonic Cancer Center, University of Minnesota. Menthofuran-treated samples were analyzed in a similar manner using an Agilent LC-MSD TOF with reflectron mass spectrometer at the Mayo Proteomics Research Center (Rochester, MN).

4.2.6 LC-ESI+-MS/MS analysis of peptides

Reversed-phase LC was performed with an Eksigent nano LC-Ultra 2D LC system (Dublin, CA) equipped with a 10-cm fused silica emitter (75 μm inner diameter from New Objective, Woburn, MA) in-house packed with reverse-phase Zorbax SB C18 5 μm resin (Agilent Technologies, Santa Clara, CA). Products were eluted at a constant flow rate of 300 nL/min with the following conditions: 25 min linear gradient from 95% A, 5% B to 80% A, 20% B; 25 min linear gradient to 10% A, 90 %B, 10 min hold at 10% A, 90% B; 5 min linear gradient to 95% A, 5% B, then a 15 min hold at 95% A, 5% B. Solvent A was 0.1% formic acid in water (v/v) and solvent B was acetonitrile containing 0.1% formic acid. ESI+-MS/MS was performed with a Thermo Scientific LTQ-Orbitrap Velos instrument (Thermo Electron, Bremen, Germany) in positive ion mode. General mass spectrometric conditions were: electrospray voltage, 1.6 kV; capillary temperature, 275 °C; no sheath or auxiliary gas flow. Protein digests (1 μL) pre-acidified with glacial acetic acid were injected onto the column. MS data were acquired with the Orbitrap analyzer at a resolving power of 60,000 at 400 *m/z*. Nine scan events were used as follows: (Event 1) *m/z* 300-2000 full scan MS and (Events 2-9) data-dependent scan

MS/MS on the eight most intense ions from event 1. An isolation window of 2.5 *m/z*, ion selection threshold of 500 counts, activation $q = 0.25$, and activation time of 30 ms were applied for MS2 acquisitions. The spectra were recorded using dynamic exclusion of previously analyzed ions for 0.5 min with two repeats and a repeat duration of 0.5 min. Ions with unassigned charge states or charge states of <2 were excluded. The MS/MS normalized collision energy was set to 35%. All data were processed with the Qual browser module of Xcalibur 2.1.0.1139 (Thermo Electron). The lock mass was enabled for accurate mass measurements. Polydimethylcyclosiloxane (*m/z*, 445.120024) ions were used for internal calibration. The mass spectrometer is located in the Analytical Biochemistry core facility of the Masonic Cancer Center, University of Minnesota.

4.2.7 Data Analysis

Tryptic digestion MS data were analyzed using Proteome Discoverer 1.3 and Xcalibur 2.1 (Thermo Electron). Peak lists and predicted CID fragmentation were generated using Proteome Discoverer, while peak areas and actual fragmentation were checked in Xcalibur. Precursor ions were required to have a mass tolerance of 5 ppm, while fragment ions were required to have a mass tolerance of 0.8 Da. Dynamic modifications were searched using Sequest on lysine, cysteine, and alanine residues (as a control) or all residues. The monoisotopic modification masses searched were: 149.09663 (addtition of menthofuran), 103.04219 (identified by mass spectra of whole, β-nicotyrine–modified P450 2A6), 173.07148 (addition of oxidized β-nicotyrine), and 189.06639 (addition of twice-oxidized β-nicotyrine). The peptide window for the peak

list was set for 300-6000 Da (singly-charged equivalent). The search database consisted of the commonly accepted sequence for P450 2A6 except for one substitution in the N-terminus (L2A) that is present in the enzyme used [110]. Xcorr scores and mass deviations were used as significance cut-offs. Since alanine cannot be modified, the highest scoring peptide with a false alanine modification was used to set the minimum Xcorr score for an adducted peptides. Once a peptide was identified, its mass was searched in all of the samples. In order for a modification to be considered valid, the peptide must: 1) have been identified by the Proteome Discoverer software using the search criteria and significance cut-offs listed above in at least one sample, 2) be present as a peak in the chromatogram and not in the controls, 3) demonstrate at least two *y*- and two *b*-ions that match with the predicted fragmentation of that modified peptide. These search criteria were selected after collaborative discussion with Dr. Martin Phillips (ORISE Fellow, FDA) who employed similar methods in his research.

4.3 Results

To investigate P450 2A6 modification by menthofuran or β-nicotyrine, the enzyme was incubated with 20 μM menthofuran or 50 μM β-nicotyrine in the presence or absence of NADPH. Two different, complementary techniques were used to detect protein modification. ESI-LC-MS was employed to determine the mass of the adduct as well as measure extent of modification. The locations of the modifications were queried through high resolution LC-MS/MS analysis of tryptic digests.

Proposed modification masses were chosen from two sources, the mass-shifts observed from LC-MS experiments of the whole and inactive enzyme (Table 4-1) and theoretical masses that were proposed based on suspected chemistry. Both menthofuran and β-nicotyrine are suggested to go through an epoxide intermediate (Figure 4-2, A and B). The proposed menthofuran chemistry mimics known furan chemistry [127].

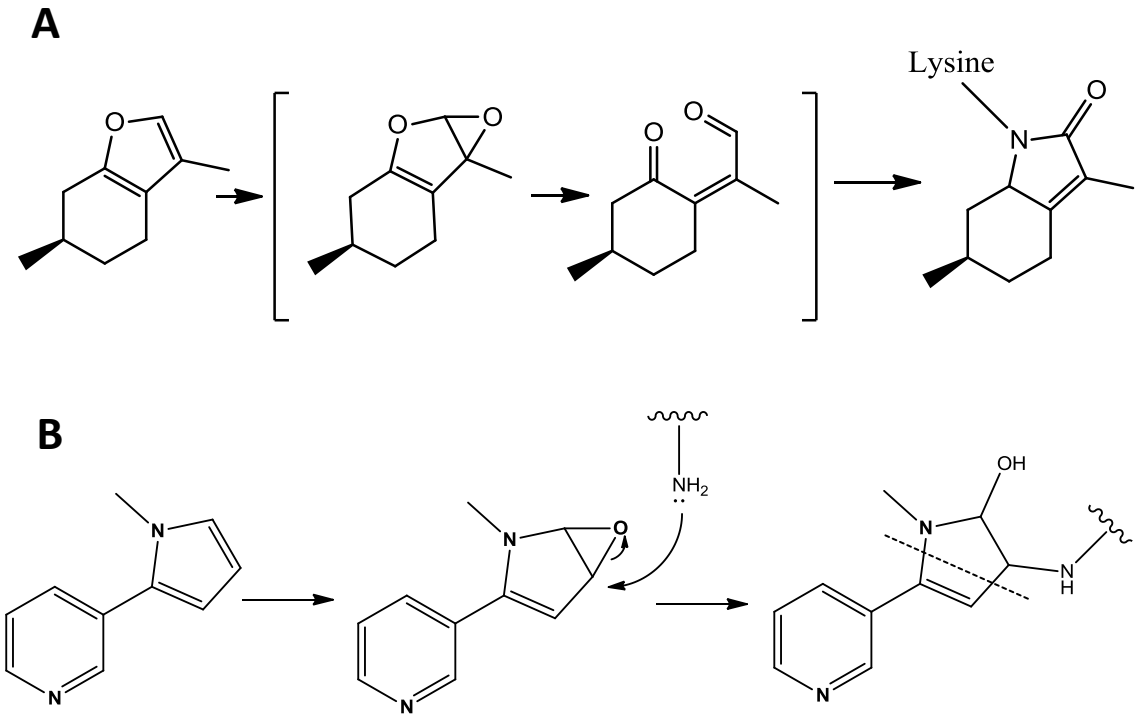


Figure 4-2. Proposed scheme for menthofuran (A) and β-nicotyrine (B) as mechanism based inactivators by lysine modification. Dotted line indicates possible fragmentation (see page 94).

4.3.1 Protein modification

ESI-LC-MS of unmodified P450 2A6 gave a mass of 57189.94 Da, which corresponds to the hypothetical mass of a menthofuran modification (Figure 4-3 A). The

menthofuran-modified sample had some unmodified P450 2A6 as shown by the peak at 57188.21 Da (Figure 4-3 C). In the same sample, evidence of adduct formation to the P450 2A6 apo-protein as seen by additional peaks in the deconvoluted spectrum. Two of these, with masses of 57205.28 and 57355.10 Da, are of particular interest (Figure 4-3, C). The first, and the most abundant peak in the deconvoluted spectrum, with a mass of 57205.28 Da gives a 17 Da mass shift. Within the mass-accuracy of this instrument, this could be the addition of water or an oxygen atom. The second, with a mass of 57355.10 Da, gives a mass shift of 150 Da which is the expected mass of a menthofuran-generated adduct. Based on the mass shift of the whole protein as well as the proposed chemistry, it is likely that the menthofuran-mediated modification has a mass of 150 Da (Figure 4-2, A). Therefore, when menthofuran-modified P450 2A6 samples were digested with trypsin and analyzed using high resolution LC-MS/MS, resulting data files were queried to look for mass shifts of 150 Da.

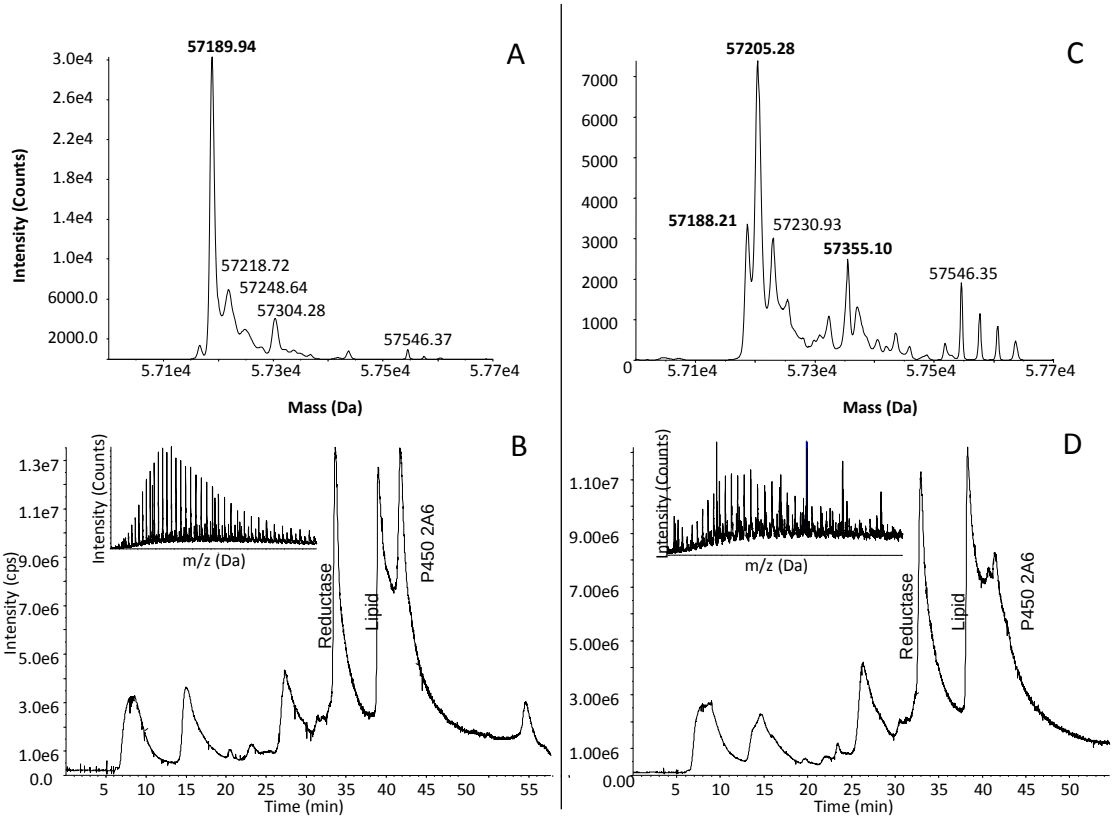


Figure 4-3. ESI-LC-MS analysis of adduct formation to the P450 2A6 protein upon inactivation by menthofuran. Reconstituted P450 2A6 was incubated with menthofuran (20 uM) in the presence (inactive sample, A and B) or absence (exposed control, C and D) of NADPH (1mM) as described in methods. Total ion chromatogram of each sample with the protein envelope for P450 2A6 shown in the inset (B and D) . Deconvoluted spectrum of the P450 peak for an exposed control sample (A) and an inactivated sample (C).

ESI-LC-MS analysis of three samples (control, β-nicotyrine and 8-MOP-modified P450 2A6 β-nicotyrine-modified P450 2A6) identified masses that corresponded to unmodified P450 2A6. In the control sample the mass was 57204.5 Da, in the β-nicotyrine-modified the mass was 57210.2 Da and in the 8-MOP-modified the mass was

57206.2 Da. This gives a variation of ± 6 Da, within the limitations of the instrument used.

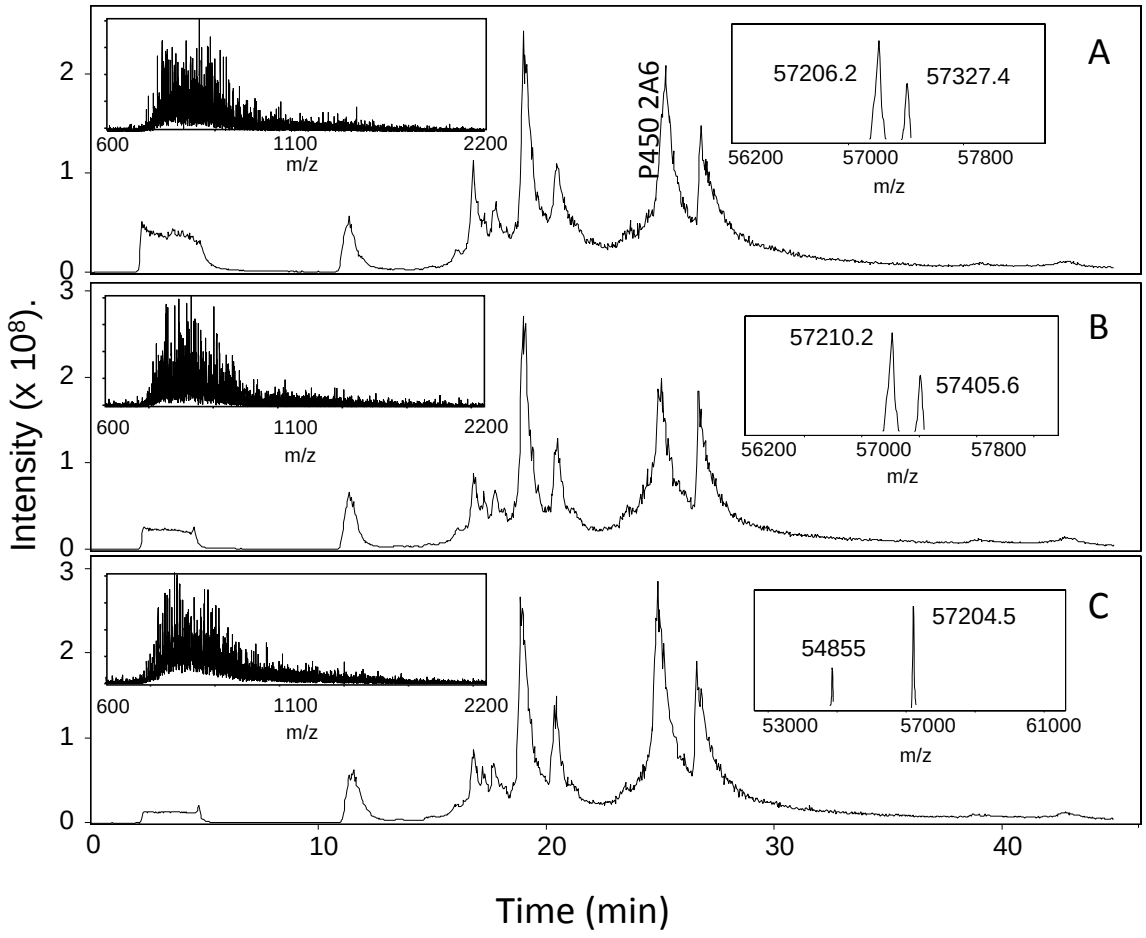


Figure 4-4. Total Ion Chromatograph from 8-MOP-inactivated P450 2A6 (A), β-nicotyrine-inactivated P450 2A6 (B) and β-nicotyrine-exposed control P450 2A6 (C). Protein envelope are shown in the left inset, deconvoluted spectra are shown in the right inset.

ESI-LC-MS analysis of β-nicotyrine-modified samples identified mass shifts from the unmodified P450 2A6 (Table 4-1). Figure 4-4, B is representative of one of the observed mass shifts with a mass of 57405.6 Da. In each of three replicates, a different mass shift was observed. Simply stated, the experiment was done three times and each

time, a different result was observed. None of the mass shifts seen were in agreement with any proposed chemistry. Likely chemistry would include a mass shift of 174 Da from addition of the β-nicotyrine epoxide intermediate (Figure 4-2, B) or perhaps a mass shift of 190 Da that could result from Michael addition of the hydroxypyrrolinone product. Therefore, when β-nicotyrine-modified P450 2A6 samples were digested with trypsin and analyzed using high resolution LC-MS/MS, resulting data files were queried to look for the mass shift observed by LC-MS as well as the proposed mass shifts based of the addition known metabolites and likely chemistry.

	Mass shifts (Da)	
	β-nicotyrine	8-MOP
Trial 1	195	121
Trial 2	106	232
Trial 3	101	337

Table 4-1. Observed mass shifts on P450 2A6 after incubation with either β-nicotyrine or 8-MOP.

An 8-MOP-mediated apo-protein adduct had been previously observed on the highly similar enzyme, P450 2A13. The previously observed 8-MOP adduct had a mass of 232 Da. Much like the results obtained with β-nicotyrine, each of the three times the experiment was performed, three different mass shifts were observed. One of the three times, the mass that was previously observed was also observed in our experiment. The two other times mass shifts of 121 and 337 Da were seen.

4.3.2 Peptide Searches

The locations of adducting fragments were searched by analyzing tryptic digests of the reaction mixtures by LC-MS/MS analysis on a LTQ-Orbitrap Velos mass spectrometer. Proteome Discoverer software was employed to determine the location of the various P450 2A6 modifications caused by treatment with menthofuran or β-nicotyrine. Unmodified peptides were identified in all samples. Coverage of the protein by unmodified peptides ranged from 67%-98% but was typically over 80%.

Table 4-2 displays results from a data query that looked for an addition of 175 Da in a sample that had been treated with β-nicotyrine. In the β-nicotyrine–P450 2A6 reaction mixtures, there was no preference, with regard to amino acid residue, for a suggested adduct. It is noted that at similar qualifying parameters, such as Xcorr and mass deviations, β-nicotyrine-modifications are suggested to appear on alanine and other residues that are unlikely to react with an electrophilic metabolite of β-nicotyrine (Table 4-2). Therefore, no adduct identifications fit the criteria outlined in the methods section. This is seen with all mass-shifts that were queried for potential β-nicotyrine-modifications as well as the mass-shift queried for menthofuran-mediated modification of P450 2A6.

Sequence	Residue and Position	XCorr	MH+ [Da]	ΔM [ppm]
DGALTQlNVAFsREQShKVYVQHLLk	L7; S12	2.52	3361.74135	-0.83
IQeEAGFLIDALR	E3	2.27	1664.86429	-0.02
vILGVTIPADSNEKHLQVIGYEKMIHhpHFSVTSIDHDImLIK	V1; P28; M40	2.13	5413.81147	1.77
lHEEIDR	L1	1.69	1085.53653	-0.87
mELFLFFTTVMQNFRLKsSQSpk	S18; P22; K23	1.67	3317.66086	-0.94
hLPGPQQQAfKELqGLEDfIAKK	H1; F10; Q14	1.51	3002.55483	0.80
GyGVVFSNgERAKQLR	G9	1.29	1971.02158	1.00
TLDPNsPRDFIDSFLIRmQEEEk	S6; K23	1.25	3144.50503	-1.47
DiDvSpK	I2; V4; P6	1.23	1295.63994	-1.54
MAaSGMLLVALLVCLTVMVLMSVwQQR	A3	1.19	3153.65805	1.94
GTevYPMlgSVLR	E3; V4; L8; G9	1.12	2118.05962	-1.61
HPEVeAkvHEEIDRVIGK	E5; K7; V8	1.03	2607.35146	1.80
kSdAFVPFSIgKR	K1; D3; G11	0.98	1974.03496	-1.81
kGQfkK	K1; F4; K5	0.97	1099.60320	-1.42
EsSfVekMK	S2; E6; K7	0.95	1622.76592	-0.81
SDAfvPfSIGK	F4; V5; F7	0.79	1689.84050	-1.15
GYGVVFSNGERAk	K13	0.78	1557.78362	1.73
NRQpKfEdR	P4; F6	0.67	1553.76372	1.85
hILAILQDCPSLRPPIDhLCELLPRLQAR	H1; H18	0.63	3520.90317	-0.05
MAaSGMLLVALLVCLTVMVLMSVWQQR	A3	0.62	3137.65866	0.52
MELFLFftTvMQNFr	F7; T8; V10	0.56	2462.18462	0.85
NRQPKFeDr	E7; R9	0.52	1537.76712	0.78
GYGVVFSNgeRAKQlr	G9; E10; L15; R16	0.50	2477.26518	1.03
GfGVGkR	K6	0.50	910.48798	-1.57
ySLdVws	W6; S7	0.50	1249.55286	0.34
hvGfATIPr	H1; V2	0.48	1219.62038	-1.23
AvKEALLDYK	V2	0.47	1323.73258	1.37
DkEFLSLLR	K2	0.46	1294.71377	-1.30

Table 4-2. Suggested 174 Da adduct sites from analysis of tryptic peptides from P450 2A6 incubated with β-nicotyrine.

4.3.3 Inactivation of P450 2A6 by ^{3}H-β-nicotyrine

Samples of ^{3}H-β-nicotyrine incubated with P450 2A6 in the presence or absence of NADPH were separated by HPLC and analyzed by Radioflow and UV. Retention time (21 min) of P450 2A6 was determined prior to the analyses (Figure 4-5). Analysis showed no radioactivity eluted with the P450 2A6 protein compared to a control (no NADPH). There was more background radioactivity seen throughout the +NADPH sample, however it accounted for less than 10% of the total radioactivity in the sample.

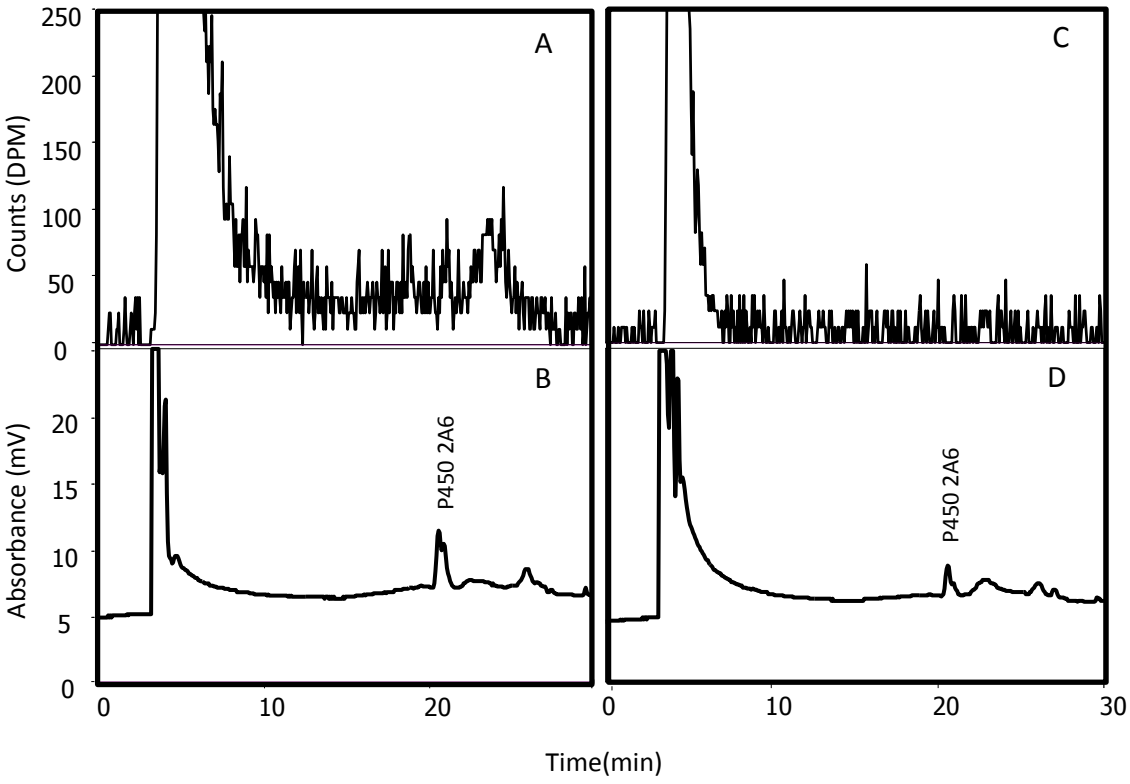


Figure 4-5. LC/Radioflow/UV analysis of P450 2A6 metabolism of with ^{3}H-β-nicotyrine in the presence (A and B) or absence (C and D) of NADPH. Radioflow traces are shown on the top (A and C) and UV traces on the bottom (B and D).

4.3.4 GSH effect on menthofuran and β-nicotyrine-mediated inactivation of P450 2A6

To trap reactive intermediates and potentially protect P450 2A6 from inactivation glutathione or semicarbazide were included in the reaction mixture. Menthofuran and β-nicotyrine concentrations were chosen so that an equal loss of enzyme activity occurred for each compound, about 60% (Table 4-3). Neither trapping agent protected P450 2A6 from inactivation by menthofuran or β-nicotyrine. However, under the conditions of these experiments (10 min) the extent of β-nicotyrine-mediated inactivation in the presence of glutathione or semicarbazide was greater than in the absence of these agents (Table 4-3). After 30 min, the extent of β-nicotyrine mediated P450 2A6 inactivation in the samples with and without glutathione were similar. Therefore, glutathione and semicarbazide appear to accelerate the rate of inactivation by β-nicotyrine. It should be mentioned that, attempts to identify a glutathione adduct were carried out with no positive results (data not shown).

Table 4-3. Effect of NADPH and trapping agents on the inhibition of P450 2A6 activity by menthofuran and β-nicotyrine

Components	Activity[b]		Amount Remaining[c]
	0 min	10 min	
	%		%
Menthofuran (5μM)	100	100	100
NADPH	91±11	81±3	90
Menthofuran (5μM) +NADPH	107±9	46±14	43
+Glutathione	115±13	56±3	48
+ Semicarbazide	104±3	42±3	40
β-nicotyrine (20μM)	100	100	100
NADPH	90±12	83±3	86
β-nicotyrine (20μM) +NADPH	89±19	32±2	35
+Glutathione	96±25	13±7	14
+ Semicarbazide	83±14	10±1	13

[a] Samples were prepared as described in the Materials and methods. Reconstituted enzyme was incubated for 10 minutes at 30 °C in the presence of 5 μM Menthofuran or 20 μM β-nicotyrine and 10 μM Glutathione or 10 μM Semicarbazide.
[b] Values are means ± S.D. from three experiments. Percent activity remaining is compared to control samples that contained no NADPH.

4.4 Discussion

The experiments presented in this chapter were designed to identify the mass of the menthofuran and β-nicotyrine-generated adduct and the site of apoprotein modification. Unfortunately, the results are largely negative or inconclusive. They are useful in some regard, and are therefore this chapter is included.

Because previous research using immunochemical methods demonstrated covalent binding of menthofuran metabolites to the P450 2A6 protein, menthofuran was used as a positive control [105]. Therefore, it was expected that a mass shift would be apparent in menthofuran-treated P450 2A6. Using a high mass-accuracy instrument, a mass shift of 150 Da was seen (Figure 4-3, C), which corresponds to the mass of the theoretical menthofuran adduct, if the menthofuran epoxide were added (Figure 4-2, A). LC-MS/MS analysis of peptides generated from menthofuran-inactivated P450 2A6 that looked for an addition of 150 Da mass shift were inconclusive. LC-MS analysis of menthofuran-inactivatd whole P450 2A6 showed a mass shift of 17 Da as the most abundant peak in the deconvoluted spectrum. The addition of 17 Da could be an oxygen, as the mass accuracy of the instrument is greater than ± 1 Da. Although the chemistry behind this result is not yet understood, it is clear that the 17 Da mass shift was dependent on NADPH and menthofuran, and is likely a byproduct of catalytic turnover of the enzyme. By comparing P450 2A6 peak in the total ion chromatograms from menthofuran-modified and control experiments, it is seen that treatment results in a smaller P450 2A6 peak (Figure 4-3). This could occur because menthofuran-modified enzyme is less soluble or because it does not ionize as well in the MS. Regardless, the quality of the results are diminished.

8-Methoxypsoralen (8-MOP) was also used as a positive control based on research in which LC-MS analysis of the adducted protein indicated an increase in the mass of 232 Da compared with the unadducted protein [71]. Under conditions that mimicked those from that paper, only one of three replicates resulted in a mass-shift of 232 Da. The other two mass-shifts (121 and 337 Da) are unlikely 8-MOP adducts and were considered false-positives. The lack of consistent results in replicates raises questions to the legitimacy of this result.

In parallel, β-nicotyrine-treated P450 2A6 was analyzed. Similar to the results with 8-MOP-treated samples, three different mass-shifts were observed. Each mass shift was observed one time of three replicates and none of these mass shifts would have resulted from the addition of any known β-nicotyrine metabolites and suggesting that these results are also false positives. One reason for the underwhelming results could be that the quality of the LC-MS data from these experiments was poor. This can be seen by the decreased P450 2A6 peak size in the TIC (Figure 4-4) and the background in the protein envelope (Figure 4-4, left inset). Although none of the mass-shifts could be replicated, the results do not exclude the possibility of an adduct on the apoprotein as the cause of inactivation.

Two of the masses, 102 and 106, were considered when querying data from LC-MS/MS analysis of peptides from β-nicotyrine-inactivated P450 2A6. If these masses are generated from a β-nicotyrine adduct, it would likely be a as a result of a fragment of thfragmentation of adduct during the ionization in the source of the mass spectrometer. Figure 4-2 shows how fragmentation across two bonds could explain the observed

masses, leaving a mass of 103 Da. The remaining two masses used while quering data from LC-MS/MS analysis of peptides from β-nicotyrine-inactivated P450 2A6 corresponded to the proposed β-nicotyrine pyrrolinone and the hydroxy-pyrrolinone adducts. These experiments were inconclusive. None of the suggested adducts were detected exclusively in β-nicotyrine-treated samples. A heme adduct is a possibility, however, experiments in Chapter 2 of this thesis that looked at heme after β-nicotyrine-mediated P450 2A6 inactivation showed no evidence of an adduct on the heme.

One final piece of evidence suggests that no covalent adduct is formed during β-nicotyrine-mediated inactivation of P450 2A6. Radiolabeled menthofuran that was incubated with P450 2A6 and analyzed by HPLC/Radioflow did not result in detectable tritium co-eluting with the enzyme. Instead, all of the radioactivity eluted at the beginning of the HPLC gradient with other small molecules. If all of the P450 was inactivated by one molecule of β-nicotyrine, a covalent modification would result in an estimated 1 million counts (dpm) and would be expected to co-elute with the P450 2A6 protein. This was calculated by multiplying the specific activity of the β-nicotyrine by the amount of P450 in the reaction (2.22×10^{12} dpm/Ci · 1Ci/mol · 100×10^{-12} mol · 90% inactivation = 1,980,000 dpm). Thus, under the conditions of this experiment, we concluede no protein adduct was formed.

Menthofuran has been shown to non-specifically bind to protein after incubation with P450 2A6, therefore a reactive metabolite gets out of the active site [105]. The possibility a reactive β-nicotyrine metabolite binding to non-specific sites on the protein could explain the inconsistent MS results. However, if this were the case, radioactive β-

nicotyrine metabolites adducted to P450 2A6 would have been detected when it was incubated with ^{3}H- β-nicotyrine. Additionally, the addition of GSH or semicarbazide did not rescue enzyme activity during inactivation experiments suggest that the reactive intermediate does not leave the active site.

Another explanation for observing inactivation without detecting a covalent adduct is that a very tight binding product is generated. In this hypothetical situation, the compound would bind the active site so tightly that k_{off}, the rate constant for release of the compound from the enzyme, would be exceedingly small. This, in effect, would produce irreversible inhibition.

4.5 Conclusion

In summary, conclusive evidence of a covalent adduct as a result of menthofuran or 8-MOP-mediated inactivation of P450 2A6 was not obtained. This does not rule out the possibility of a covalent adduct, however confirmation would require further experiments. In the case of the β-nicotyrine-mediated inactivation of P450 2A6, the evidence presented here supports the conclusion that no covalent adduct is generated.

Chapter 5

Comprehensive Conclusion

In this thesis the inhibition, inactivation and metabolism of a handful of compounds by P450 2A6 and P450 2A13 were studied. The overarching goal of these studies was to elucidate the mechanism by which nicotine metabolism leads to the inactivation of P450 2A6 and P450 2A13. β-Nicotyrine had been suggested to be an inactivator of P450 2A6 and some evidence suggested that it is a product of nicotine metabolism *in vivo* [90]. Thus, we hypothesized that β-nicotyrine could be a metabolite of nicotine that leads to inactivation of P450 2A6 and P450 2A13.

For this hypothesis to be true, β-nicotyrine would have to be a P450 2A6 and P450 2A13-mediated nicotine metabolite and β-nicotyrine metabolism catalyzed by these enzymes would result in their inactivation. In Chapter 2, mechanism based inactivation of P450 2A6, but not P450 2A13 occurred during incubations with β-nicotyrine. Then, in Chapter 3, we show that β-nicotyrine is generated in a time and concentration-dependent manner during P450 2A6-mediated metabolism of nicotine. Under the same conditions, P450 2A13-mediated metabolism of nicotine did not generate β-nicotyrine. Based on these two results, in can be concluded that β-nicotyrine could be involved in the inactivation of P450 2A6 but not P450 2A13. When inactivation of P450 2A6 and P450 2A13 during nicotine metabolism was first observed, it was assumed that the inactivation

of these two enzymes occurred by the same mechanism. These results, however, suggest that it is possible two different mechanisms are responsible for the inactivation of P450 2A6 and P450 2A13 during nicotine metabolism.

The hypothesis that the inactivation resulting from P450 2A6-mediated β-nicotyrine metabolism was due to a covalent adduct to the enzyme was not supported by any of the experiments presented in this thesis. The results in Chapter 4 indicate that a covalent adduct to the apoprotein is not formed and Chapter 3 detected no adduct to the heme. As discussed in Chapter 4, an explanation for the observed inactivation without covalent binding would be a very tight binding product. Upon review of literature that describes amino acid side-chains that surround a molecule that is in the P450 2A6 active site, there are no apparent sites for adduction. Residues surrounding the active site are hydrophobic in nature, with the exception of an asparagine at 297 [70]. Chemically, the inactivation happening through a very tight-binding intermediate, rather than a covalent bond, makes much more sense.

To further elucidate the mechanism by which β-nicotyrine metabolism leads to P450 2A6 inactivation, its metabolism was characterized. Three metabolites of β-nicotyrine are identified and described in Chapter 3. The results are in agreement with previous research that identified products of β-nicotyrine metabolism by rabbit liver microsomes. The kinetic parameters of initial β-nicotyrine oxidation are estimated. The K_m is similar to that of P450 2A6-mediated coumarin metabolism and the V_{max} is five times higher. Coumarin is considered a good substrate for P450 2A6, β-nicotyrine is better and it is readily metabolized by this enzyme.

P450 2A13-mediated β-nicotyrine metabolism generated the same three products as did P450 2A6. Additionally, the kinetic parameters for P450 2A13 oxidation of β-nicotyrine were nearly the same as for P450 2A6. These results rule out distinct metabolites being formed or rate of metabolism being the reason that P450 2A6, but not P450 2A13, is inactivated during β-nicotyrine metabolism. Remaining explanations for the difference between the two enzymes point toward the larger size and more permissive geometry of the P450 2A13 active site. This explanation fits well with the concept of a very tight, but non-covalent binding inactivating species. In the P450 2A13 active site, there is essentially more room for the molecule to move around and possibly escape the active site.

Ultimately, the research presented in this thesis is mechanistic and many steps removed from having an impact on human health. However, results presented here can start to shape our understanding of whether or not β-nicotyrine could modify nicotine metabolism *in vitro*. The amount of β-nicotyrine found in the smoke from one cigarette is low (25-250 nmol) compared to the nicotine (5-14 μmol), and the dissociation constants for both of these enzymes with P450 2A6 are similar (Chapter 2) so the nicotine would likely outcompete β-nicotyrine for access to the active site. However, a novel finding that is presented in this thesis is that P450 2A6-mediated nicotine metabolism generates β-nicotyrine. β-nicotyrine, then, is already in the active site. Since we have shown that β-nicotyrine is readily metabolized by P450 2A6, it would likely be further metabolized to the species that inactivates the enzyme.

In Chapter 3, we measured the amount of β-nicotyrine generated during P450 2A6-mediated nicotine metabolism. This amount, however, is likely not representative of the true amount of β-nicotyrine nor is it useful to calculate rate constants because it is likely that as β-nicotyrine is generated, it is metabolized. To accurately assess activation potency, an experiment quantifying all β-nicotyrine metabolites as well as inactivated protein should be carried out to calculate the appropriate kinetic parameters (Figure 5-1). This type of experiment has limitations. To determine kinetic parameters for each step, metabolites must be quantified at very short times while concurrently measuring enzyme inactivation. To add to the complexity, it would also be necessary to distinguish between sequential metabolism and metabolism that might occur after a metabolite dissociates and then reenters the active site of the enzyme.

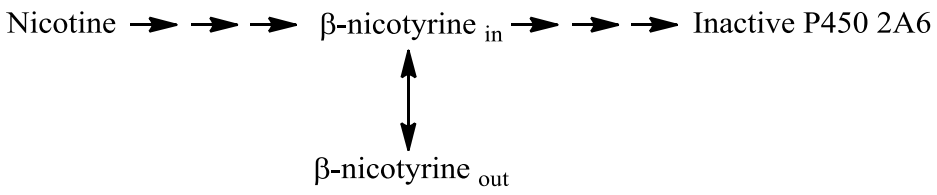


Figure 5-1. Nicotine metabolism to β-nicotyrine and P450 2A6 inactivation.

Regardless of experimental difficulty, β-nicotyrine is a good candidate for further research with the long term goal of using it for smoking cessation. We have shown that it does inactivate P450 2A6, and previous studies in which smokers are administered 8-MOP have shown reduced smoking in a laboratory setting[88]. As discussed in the

introduction to Chapter 4, mechanism-based inactivators make particularly potent drugs which allow for decreased dosing and subsequently less likelihood for harmful side effects [125]. Enzyme-adduct covalent complexes, however, have been shown to cause immune reactions leading to idiosyncratic adverse drug reactions. Because we have shown evidence of a very tight-binding, but not covalent, inactive complex, this makes this compound that much more favorable for development toward a drug.

A necessary next step would be to determine with human liver microsomes if β-nicotyrine is a substrate for other P450 enzymes, if it inactivates any of those P450s and if it is metabolized by any other families of enzyme involved in xenobiotic metabolism. Additionally, published work suggests that β-nicotyrine does not have the addictive properties that nicotine does, and would therefore not contribute to dependence, however further research in this area would be needed.

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